The impact of plant chemicals on bee health: Interactions with parasites and immunity

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Declaration of authorship: I Arran James Folly hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others this is clearly stated.

Signed:

Date:

For Robert Budge,
who started my fascination
with the natural world.

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Buff-tailed bumblebee (*Bombus terrestris*) queen foraging in Windsor Great Park (photo credit: A. J. Folly 2018)

"Can a bee be said to be, or not to be, an entire bee,
when half the bee is not a bee?"

(Monty Python 1972)

Abstract

Wild bumblebee populations are under increasing threat from both a reduction in natural floral resources, as a direct result of anthropogenic disturbance, and an increase in disease prevalence, including emergent infectious diseases. This is of global concern as bumblebees are not only valued for their economic importance as pollinators but also culturally, as a charismatic component of the natural world.

This thesis explores the impacts of phytochemicals found in the pollen and nectar of plants included in Agri-environment scheme (AES) planting strategies on bumblebee health, with a focus on their interactions with prevalent parasites. The five research chapters investigate the impact of a range of phytochemicals on two key bumblebee parasites, using a range of approaches including *in vitro* cellular growth experiments, *in vivo* experiments in individual bumblebees, both as larvae and adults, and finally epidemiological experiments on whole bumblebee colonies.

In chapter one I review the pertinent literature relating to bumblebee declines and the impact of phytochemicals on pollinator health. In chapter two, larval inoculation with *Crithidia bombi* (Trypanasomatidae) resulted in no infected larvae seven days following inoculation. This result was critical for the design of subsequent chapters that tested the impact of phytochemicals on larval stages. In addition larvae were identified as disease transmission hubs (chapter published as *Folly et al. 2017 Journal of Invertebr Pathol*). Chapter three describes the identification of 62 unique phytochemicals from the pollen and nectar of AES plants. In addition, chapter three investigated the impact of four of these AES phytochemicals with known biological activity on *C. bombi in vitro*. Here caffeine had a significant positive effect on the growth of *C. bombi* at its ecologically relevant concentration before significantly reducing *C. bombi* growth at higher concentrations. In chapter four I designed a proof of principle investigation to ascertain if phytochemicals could impact *Nosema bombi* (Microsporidia) infection in *B. terrestris* using the isoflavone biochanin A. Biochanin A had a significant prophylactic effect in developing larvae and a significant therapeutic effect in infected adult workers. In chapter five, the phytochemicals caffeine and tricoumaroyl spermidine, which were found in nectar and pollen from

AES plants (Chapter three), were tested at their ecologically relevant concentrations against *N. bombi* in larvae, both prophylactically and therapeutically. Caffeine had a significant prophylactic and therapeutic effect on *N. bombi* infection intensity. These novel results clearly show that phytochemicals can impact on *N. bombi* infections in bumblebees. In chapter six, caffeine was continuously fed to wild caught and reared *B. terrestris* colonies that were infected with *N. bombi*. Caffeine reduced the overall colony infection prevalence of *N. bombi*. In addition those bumblebees that were infected had significantly lower infection intensities. This chapter represents the first evidence of the impact of phytochemicals on disease epidemiology in bumblebees. Finally in the discussion chapter I outline the wider reaching implications of my research and discuss how my findings may impact the direction of future research in this area.

Overall I found that floral rewards may contain a diverse suite of phytochemicals. Given that bumblebees undertake numerous foraging trips it is likely that a developing colony is consistently exposed to such phytochemicals. Importantly some of these may have positive impacts on bumblebee health by mitigating the impact of diseases. These findings have important implications for both conservation practices and government policy makers.

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Chapter 1

Introduction

1. Introduction

1.1 The impact of plant chemicals on bee health: Interactions with parasites and immunity

The corbiculate Apinae (pollen basket bees) are an economically important guild of insect pollinators (Wilson 1971, Gallai *et al.* 2009). Within this guild, wild pollinators such as bumblebees (genus *Bombus*) provide essential pollination services (Williams 2003, Breeze *et al*. 2011). Some of these charismatic pollinators are in sharp global decline (Fitzpatrick *et al*. 2007, Williams & Osborne 2009, Cameron *et al*. 2011, IUCN 2015) and a number of contributing factors to this decline have been outlined. These include the use of pesticides (Gill *et al*. 2012, Whitehorn *et al*. 2012, Siviter *et al*. 2018), an increase in parasite prevalence (Cameron *et al*. 2011, Fürst *et al*. 2014) and a reduction in wildflower meadows, linked to the intensification of agriculture (Ollerton *et al*. 2014). As yet, no single factor has been shown to be the main driver of declines in bumblebee populations, and it is becoming evident that an interaction between factors is likely to be driving the observed declines (Vanbergen *et al*. 2013, Goulson *et al*. 2015).

Initiatives have been established in an attempt to mitigate the effect of intensive agricultural practices on floral diversity and keystone insect pollinators. Agri-Environment Schemes (AES) (Natural England 2017) and Conservation Reserve Programs (CRP) (USDA Farm Service Agency 2016) financially reimburse landowners that manage their land in a way that is deemed beneficial for wild animal populations (Haaland *et al*. 2011, Batáry *et al*. 2017). AES recommend the use of set flower mixes, which aim to increase floral diversity and support wild pollinator populations (Natural England 2015). An increase in floral diversity and abundance in homogeneous landscapes is beneficial for wild insect pollinators (Carvell *et al*. 2007, Wood *et al*. 2015, Carvell *et al*. 2017) and there is experimental evidence that wildflower strips and field margins, two prescriptions set out by AES, actively increase both bumblebee density and pollination services (Lye *et al.* 2009, Feltham *et al*. 2015). Interestingly however, the effect of the diverse floral biochemistry that these schemes generate on bumblebee health has received relatively little attention.

An emerging field of ecology is investigating the impact of such floral chemistry on insect pollinators (Baker & Baker 1975, Baker 1977, Adler 2000, Wright *et al*. 2013, Richardson *et al*. 2015, Stevenson *et al*. 2017). Plant secondary metabolites are synthesized in plant tissues and are classified as any compound that is not used for primary development. These metabolites are translocated, albeit in lower concentrations, when compared with vegetative plant tissues, into floral rewards (Adler 2000, Cook *et al*. 2013) and may have a negative impact on pollinators (Arnold *et al*. 2014, Tiedeken *et al*. 2016). However, plant metabolites are known for their antimicrobial properties (Cowan 1999) and consequently some of these bioactive compounds may provide indirect health benefits to pollinators by reducing parasite loads (Manson *et al*. 2010, Richardson *et al*. 2015, Giacomini *et al*. 2018).

Motivated by these implications for bumblebee health, this introduction to my thesis addresses the threats to wild bumblebee populations and the current mitigating measures that are in place to support the economically important pollination services they provide. Primarily, however, this introduction focuses on the current knowledge of the interaction between floral biochemistry and its putative impact on bumblebee health. If there is evidence of health benefits to pollinators from phytochemicals, strategies that increase floral abundance and diversity could use floral chemistry as a proxy for plant selection to support the health of wild pollinators.

1.2 Global biodiversity

There are an estimated 8.7 million species that inhabit the Earth (Mora *et al*. 2011). However, this global biodiversity may currently be undergoing a sixth mass extinction (Barnosky *et al*. 2011) and consequently this incredible diversity may be impossible to comprehensively catalogue (Costello *et al*. 2013). The current trends in global biodiversity declines are linked to a reduction in natural heterogeneous habitat, due in part to anthropogenic stressors, including an increase in intensive agriculture (Krebs *et al*. 1999, Donald *et al*. 2001, Ollerton *et al*. 2014), and an increase in disease prevalence, including a rise in emerging infectious diseases (EIDs) (Daszak *et al*. 2000, Jones *et al*. 2008). The consequences of declines in global biodiversity, such as the loss to ecosystem services, are well documented (Chapin *et*

al. 2000) especially so for economically important insect pollinators, such as bumblebees (Biesmeijer *et al*. 2006, Potts *et al*. 2010).

1.3 Bumblebees

Bumblebees are generalist pollinators that undergo an annual lifecycle (Sladen 1912, Wilson 1971, Alford 1975). They typically form small, relatively simple eusocial colonies that are initiated by a single queen following hibernation (Wilson 1971). Bumblebees not only have huge cultural significance (Shakespeare 1609, Rimsky-Korsakov 1899, Rowling 1999) but they also provide essential, economically important pollination services (Breeze *et al*. 2011, Kleijn *et al*. 2015). The corbiculate bees (Apinae), which include bumblebees, are entirely dependent on the pollen and nectar of angiosperms for nutrition. Nectar is typically carbohydrate rich and provides energy, whilst pollen has a high protein content essential for larval development (Wilson 1971). Bumblebees have special adaptations to help collect these essential floral resources including a hairy body and a sclerotised region on the hind tibia of females, known as a pollen basket, which can be used for transporting pollen. During foraging trips bumblebees may deposit pollen onto receptive floral stigma, which indirectly makes them effective pollinators (Breeze *et al*. 2011, Kleijn *et al*. 2015). However, there is compelling evidence to suggest that bumblebee populations are declining globally (Williams 1982, Fitzpatrick *et al*. 2007, Cameron *et al*. 2011, IUCN 2015), and that these declines will have a cascading effect on both plant reproductive success (Lach *et al*. 2015) and economically important crop yields (Gallai *et al.* 2009, Breeze *et al*. 2011, Kleijn *et al*. 2015).

1.4 The impact of parasites on bumblebees

A contributing factor linked to the reported global decline in bumblebees is an increase in parasite prevalence (Cameron *et al*. 2011, Fürst *et al*. 2014). The high population densities and low genetic variability associated with social insects are ideal for pathogen transmission (Schmid-Hempel 1998, Barribeau *et al*. 2015, but see also Van

Baalen & Beekman 2006). During their natural colony lifecycle bumblebees are exposed to a diverse array of parasites (Schmid-Hempel 1998), which may have negative impacts on the colony (Brown *et al*. 2003, Otti & Schmid-Hempel 2008). Two parasite species that have received much attention are the trypanosome, *Crithidia bombi* (Gorbunov 1987) and the microsporidian, *Nosema bombi* (Fantham & Porter 1914). Both have been shown to have negative impacts on their bumblebee hosts (Brown *et al*. 2000, Brown *et al*. 2003, Otti & Schmid-Hempel 2008, Rutrecht & Brown 2008, Schlüns *et al*. 2010). However, virulence in and of itself cannot explain the impact of a parasite on host populations, as parasite prevalence is a co-determinant of impact. Consequently, it remains unclear whether the recorded increase in bumblebee parasite prevalence contributes to the regulation of local bumblebee populations or if it may have more severe and wider reaching implications such as permanently reducing geographic range or species richness.

Crithidia bombi is an internal parasite that is transmitted either vertically (transovariliy) or horizontally (between conspecifics) and infects adults *per os* (Schmid-Hempel & Schmid-Hempel 1993, Otterstatter & Thomson 2007). The infection is extra cellular and found in the midgut lumen and rectum (Schmid-Hempel 1998). *C. bombi* has been shown to reduce the success of colony founding in infected queens (Brown *et al*. 2003) and infected workers experience a higher mortality rate under stressful conditions (Brown *et al*. 2000). The typical prevalence of *C. bombi* in wild bumblebee queens is 5-18% (e.g., Jones & Brown 2014). Later in the season as colonies grow and more interactions with conspecifics occur, prevalence in workers may increase to between 50-100% (Shykoff & Schmid-Hempel 1991, Imhoof & Schmid-Hempel 1999). In addition to this, the global prevalence of *C. bombi* is rising. The transportation of commercial bumblebee colonies for pollination services has introduced *C. bombi* to South America where it is presumably increasing its geographic range (Schmid-Hempel *et al*. 2014), and is linked to declines in native bumblebees (Schmid-Hempel *et al.* 2014). However, current understanding assumes that this parasite only infects adult bumblebees, but it remains unclear whether larvae can become infected with *C. bombi*, and how this may impact parasite prevalence and colony fitness. Larvae are a critical component of colony development and its subsequent reproductive success. Consequently diseases that affect the brood may have more severe fitness consequences in comparison to diseases which only impact adult bumblebees.

Similarly to *C. bombi*, *N. bombi* is presumably transmitted by shared contact with conspecifics (van der Steen 2008). This microsporidian parasite is estimated to have a lower prevalence than *C. bombi* in wild populations, typically ranging between 0-9% in adult bumblebees (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014). However, *N. bombi* arguably has higher virulence when compared to *C. bombi* (Otti & Schmid-Hempel 2008, Rutrecht & Brown 2009). Consequently, *N. bombi* may be a more important parasite to investigate in regard to bumblebee declines. In contrast to *C. bombi*, *N. bombi* is known to infect bumblebee larval stages (Fantham & Porter 1914, Rutrecht & Brown 2007). Infection here occurs *per os*, presumably through trophallaxis, and manifests itself in the gut, malpighian tubules and fat bodies of its host (Fantham & Porter 1914, Schmid-Hempel 1998). Following eclosure *N. bombi* infection persists within adult bumblebees (Rutrecht & Brown 2007) and intra- or intercolonial transmission may then occur (van der Steen 2008). Work by Rutrecht & Brown (2008) showed that infection success in *B. lucorum* colonies was highest when larval exposure to *N. bombi* was high using parasite-contaminated pollen pellets as an inoculation source. However, this method, whilst successful for inoculating bumblebees, does not provide quantifiable individual inoculation, which is essential in determining both the virulence of a microbial pathogen (Brown *et al*. 2000) and also its infection success (Ruiz-González & Brown 2006).

Infection with *N. bombi* may be more deleterious to bumblebee populations when compared with other prevalent parasites. Infected *B. terrestris* colonies that were observed during a field experiment had significantly smaller colony sizes and produced no sexual offspring (Otti & Schmid-Hempel 2008). As described above, bumblebee colonies are annual and only mated gynes persist through the winter. Consequently, if infection with *N. bombi* reduces overall colony size and the production of sexual castes it may also directly impact on the reproductive fitness of its host. More specifically, bumblebee family lineages may not persist to the next generation, which may lead to localised population bottlenecks and ultimately a reduction in genetic diversity. Whilst most European studies investigated the impact of *N. bombi* on *B. terrestris*, it is important to note that *N. bombi* can infect multiple *Bombus* hosts and has variable impacts (Rutrecht & Brown 2009). In addition, the population declines and range collapses of a number of charismatic North American bumblebees have been linked to *N. bombi* infection (Cameron *et al*. 2011, Cameron *et al*. 2016). Within North America *N. bombi* is an emerging infectious disease (EID), presumably introduced from Europe

in commercially produced *B. terrestris* colonies (Cameron *et al*. 2011). Historical high prevalence of *N. bombi* has been linked to a reduction in genetic diversity and it is likely that these two factors are interacting and are currently considered reliable predictors of North American bumblebee declines (Cameron *et al*. 2011, Cameron *et al*. 2016).

As noted above, both *C. bombi* and *N. bombi* are transmitted *per os* and evidence has been found to suggest that communal areas used by hetero-colonial conspecifics and other hetero-specific pollinators, such as floral surfaces, may be important facilitators of pathogen transmission (Durrer & Schmid-Hempel 1994, van der Steen 2008, Ruiz-Gonzalez *et al.* 2012,). Consequently, as bumblebees regularly come into contact with each other and with other pollinators, which may carry disease (Fürst *et al*. 2014), at these sites, it is critical that they can mitigate the impact of infection. Bumblebees have potent innate antimicrobial immune defenses (Marmaras *et al*. 1996, Haine *et al*. 2008) which when activated by *C. bombi* infection have been shown to reduce parasite load via the upregulation of antimicrobial peptides (Brunner *et al*. 2013). These innate immune defenses can be complemented by horizontally transmitted, beneficial gut microbes (Koch & Schmid-Hempel 2011) which, when present have resulted in a lower likelihood of *C. bombi* infection. In addition, there is behavioural evidence to suggest that bumblebees can recognize and avoid artificial flowers that have *C. bombi* present, albeit in excess of ecologically relevant concentrations (Fouks & Lattorff 2011). In addition to the individual immune responses outlined above, bumblebees have also evolved social immune mechanisms, termed social immunity (Cremer *et al*. 2007). Behaviours such as corpse removal (termed necrophoresis), may allow a colony to protect itself from infectious diseases by removing compromised individuals, although preliminary work suggests that this is not an effective mechanism in the case of *N. bombi* (Munday & Brown 2017). Despite these adaptations to mitigate the impact of their internal parasites, pathogen prevalence in the bumblebee community is rising (Cameron *et al*. 2011, Fürst *et al*. 2014) and alongside potentially contributing to population declines, this has very real implications for the economically important pollination services that bumblebees provide.

1.5 Agri-Environment Schemes (AES) and wild pollinators

A large proportion of economically important crops are entomophilous (Aizen *et al*. 2009, Breeze *et al*. 2011) and pollination services provided by bumblebees, and other pollinators, are essential to maintain the increasing demand of global food production (Aizen *et al*. 2009, Tilman *et al*. 2011). Agricultural intensification and urban expansion has led to a depauperate assemblage of wild flowers where once there was diverse and abundant forage for bee pollinators (Couvillon *et al*. 2014, Goulson *et al*. 2015). A reduction in floral diversity leading to a monotypic diet has been identified as a putative cause of bumblebee declines (Goulson *et al*. 2005, Ollerton *et al*. 2014). AES and CRP attempt to mitigate the effect of intensive agriculture on wild animal populations by increasing floral diversity and abundance (Natural England 2017, USDA Farm Service Agency 2016). Wild honeybees have shown a visitation preference towards AES land (Couvillon *et al*. 2014), which has also been observed in bumblebees (Pywell *et al*. 2006, Carvell *et al*. 2007, Wood *et al*. 2015). This preference has now been linked to a direct fitness benefit for bumblebees, as landscapes with improved floral diversity and abundance have been shown to increase bumblebee family lineage survival (Carvell *et al*. 2017).

The parameters of AES prescriptions vary marginally across Europe (Haaland *et al*. 2011, Batáry *et al*. 2015). Within England approximately 6.4 million hectares or 70% of agricultural land is currently under AES, although only a small proportion of this is explicitly managed for biodiversity (DEFRA 2015). Two common prescriptions for pollinators under AES in England are the use of wildflower strips and field margins. The use of these prescriptions increases pollinator visits to soft fruit crops (Feltham *et al*. 2015) and may provide adequate foraging resources and nesting habitats for bumblebee queens (Lye *et al.* 2009). Alongside wildflower strips and field margins, pre-existing resources such as hedgerows have been shown to provide important forage for wild bumblebees (Hanley & Wilkins 2014, Batáry *et al*. 2015) and these must be included when assessing the availability of foraging resources within an agricultural setting.

Natural England, a governmental entity, determines the flowers that are to be used in AES (Natural England 2015) and these prescriptions have been developed with the scientific community (Dicks *et al*. 2015). Wildflower strips rich in legume species have been recorded to attract the highest diversity of bumblebees, including rare

species such as *B. ruderatus* and *B. muscorum* (Haaland *et al.* 2011). Evidence for legume species attracting a higher diversity of bumblebee pollinators has also been identified in arable field margins (Carvell *et al*. 2007), with an estimated 269 times more bees observed in legume based pollen/nectar mix. Plants of the Fabaceae family have been identified as a major source of pollen for most bumblebees (Goulson *et al*. 2005) but promoting legume-based seed mix AES schemes may also inadvertently be restricting the availability of other diverse bee forage in agricultural environments. Increased visitation records (Carvell *et al*. 2007, Haaland *et al.* 2011, Feltham *et al*. 2015) do not necessarily mean that bumblebee diversity is improving or that annual colony founding by queens is increasing. Wildflower strips could be acting as a sink for local species when encountering limited floral diversity, and the presence of legumes affecting bumblebee abundance may be merely an artifact. Interestingly, experimental work has now monitored gene frequencies over generations to show that florally enriched landscapes do have a positive fitness benefit for bumblebees (Carvell *et al*. 2017). However, whether this fitness benefit is a direct consequence of legumes or if it extends to other pollinators remains to be seen.

Wild bees provide essential pollination services but it has been found that a limited subset of common species provide this service and that rare species are observed less often as crop pollinators (Kleijn *et al*. 2015). Nevertheless, if the aim of agri-environment interventions is to maintain broader pollinator biodiversity, seed mixes must be tuned to benefit all insect pollinators and thus to increase pollinator diversity in an ecosystem, not just support generalist pollinators. Restrictive diets, which may be encountered in agricultural land, especially those with low nutrient quality and diversity are not beneficial for bumblebees especially if they contain detrimental secondary metabolites (Adler 2000, Arnold *et al*. 2014, Stevenson *et al*. 2017). While some of these bioactive compounds have indeed been shown to have negative effects on pollinators (Arnold et al. 2014, Nepi 2014, Tiedeken *et al*. 2016), interestingly antimicrobial properties have been identified in some secondary metabolites that may have indirect positive health benefits for the pollinators that consume them (Cowan 1999, Manson *et al*. 2010, Richardson *et al*. 2015). Consequently determining the impact of plant biochemistry on pollinator health is critical to comprehensively evaluate the effectiveness of AES and other schemes that increase floral abundance and diversity.

1.6 Bioactive compounds in floral rewards and their effect on pollinators

Floral rewards are the primary attractant for insect pollinators, however as described above, these may contain secondary metabolites (Baker & Baker 1975, Adler 2000, Stevenson *et al*. 2017). The concentrations of secondary metabolites in floral rewards are notably lower than in vegetative tissues, and within floral rewards concentrations in pollen can be up to 100 times more than in nectar (Adler 2000, Cook *et al*. 2013, Lohaus & Schwerdtferger 2014). Pollen provides protein, which is used by a bumblebee colony for the growth and development of larvae (Wilson 1971, Rotheray *et al*. 2017). Consequently, the higher concentration of phytochemicals found in pollen may have a stronger impact on the health of the developing brood when compared to adult bumblebees, which may be important for brood diseases and colony fitness more generally. However, despite this, the biochemistry of nectar has received the greatest scientific attention (Adler 2000, Manson *et al*. 2010, Wright *et al*. 2013, Lohaus & Schwerdtferger 2014). Nectar is excreted from glands called nectaries located on floral or extrafloral tissues and its composition is approximately 90% aqueous sugars, with the remaining 10% being made up of amino acids, lipids, antioxidants, mineral ions, peptides and secondary compounds (Adler 2000, Heil 2011). Interestingly, the relative concentrations of nectar phytochemicals can be affected by leaf herbivory and a plants access to nutrients, suggesting that concentrations in reproductive and vegetative tissues are not truly independent (Adler *et al*. 2006, Lucas-Barbosa *et al*. 2011). In addition, nectar chemistry varies intraspecifically across geographic space and between different crop varieties (Egan *et al*. 2016, Egan *et al*. 2018, respectively). Given that nectar chemistry shows such variation, it is likely that any impact of phytochemicals in floral rewards on pollinators may also vary depending on the species of angiosperm encountered by a forager and its geographic location.

The presence of secondary metabolites in nectar has been suggested to increase plant fitness by improving pollinator fidelity, pollen transfer and by preventing nectar robbing and microbial growth (Adler 2000, Heil 2011). However, with its high sugar content, nectar already has inherently strong antimicrobial properties due to extreme osmotic stress (Adler 2000, Erler 2014). In addition, the nectar redox cycle produces high levels of hydrogen peroxide, which may protect against nectar-based pathogens (Carter & Thornburg 2004). These factors result in an

unsuitable environment for many floral pathogens. However, whilst secondary metabolites in nectar may improve plant fitness Adler & Irwin 2005, Thomson *et al*. 2015) they may also have indirect negative fitness consequences for the pollinators that consume them (Arnold *et al*. 2014, Tiedeken *et al*. 2016). Consequently by translocating secondary metabolites to floral rewards, entomophilious plants are presented with a fitness trade off (Gegear *et al*. 2007, Cook *et al*. 2013), as pollinator fidelity is critical in maintaining reproductive fitness in angiosperms (Brosi & Briggs 2013).

Angiosperms may manipulate pollinator behaviour to increase their own reproductive success using phytochemicals (Kessler *et al*. 2008). Alkaloids are one such, naturally occurring organic compound, which may be produced by a plant, typically for use in defence. The impact of alkaloids on pollinators has been well researched (Wink 1993, Chittka *et al*. 2003, Gegear *et al*. 2007, Wright *et al*. 2010), most likely due to their use in human medicines and natural products (Cowan 1999). One floral alkaloid that has been shown to impact pollinator behaviour is caffeine. Caffeine is a methylxanthine alkaloid and has been identified in nectar using liquid chromatography and mass spectrometry (LC-MS) (Kretschmar & Baumann 1999). Caffeine consumption can potentiate the responses of mushroom body neurons in honeybees (Wright *et al*. 2013). These receptors are involved in olfactory learning and memory formation and consequently entomophilous plants may increase their reproductive success by maintaining nectar caffeine at undetectable threshold levels to enhance the memory of reward to a pollinator. Caffeine can also manipulate recruitment behaviour in honeybees, even if nectar is substandard, resulting in sub-optimal foraging (Couvillon *et al*. 2015). Caffeine synthesis has evolved independently in angiosperms five times (Huang *et al*. 2016). Consequently, caffeine may have a significant evolutionary role for angiosperms, either as a herbivore deterrent (Bernays *et al*. 2000) or as a pollinator manipulator (Wright *et al*. 2013). In addition caffeine has antimicrobial properties (Raj & Dhalla 1965) and may therefore provide indirect positive health benefits to pollinators through the mitigation of disease (see below).

One function of plant alkaloids is to evoke a deterrent response (Detzel & Wink 1993). At high concentrations they can be lethal to pollinators (Kohler *et al*. 2012) and can manipulate pollinator behaviour as described above (Wright *et al*. 2013, Couvillon *et al*. 2015). However, these findings are often a result of using concentrations in excess of ecologically relevant ecological values. Bumblebees are not deterred or

responsive to ecologically relevant concentrations of *Rhododendrum ponticum* nectar toxins, which may be due to low levels of gustatory receptors (Tiedeken *et al*. 2014). However, when encountering alkaloid concentrations in excess of ecological relevance in nectar, bumblebees show a behavioural propensity to select artificial flowers with lower concentrations of nectar alkaloids (Gegear *et al*. 2007). Consequently, excessive alkaloid concentrations may be detectable and therefore avoided by bumblebees. Therefore, to provide fitness benefits to a plant the concentration of nectar alkaloids may need to fall below a pollinators threshold detection level.

Bumblebees, similarly to other insect pollinators are able to mitigate the effect of some plant secondary compounds, such as alkaloids, using enzyme based metabolic pathways to reduce their toxicity (Després *et al*. 2007). Consequently bumblebees may have adapted to cope with ecologically relevant levels of nectar toxins. Furthermore any negative effects on individuals as a result of nectar alkaloid consumption depend on the dominance status of adult bumblebees (Manson & Thomson 2009). Interestingly the effect of secondary metabolites in pollen, the primary protein source for bumblebee larvae (Wilson 1971), has received less attention even though phytochemicals may be encountered at higher concentrations in pollen and are therefore more likely to elicit a pharmacological response (Kretschmar & Baumann 1999, Cook *et al*. 2013).

Due to the relatively high nutritional requirements of a developing bumblebee colony (Rotheray *et al*. 2017) it is likely that a colony is consistently exposed to phytochemicals. Consequently bioactive phytochemicals may play a central role in maintaining colony health and may interact with pollinator disease epidemiology. The impact of phytochemicals on bumblebee health, including interactions with *C. bombi* has been investigated, both *in vitro* (Palmer-Young *et al*. 2016, Palmer-Young *et al*. 2017) and *in vivo* (Manson *et al*. 2010, Richardson *et al*. 2015, Giacomini *et al*. 2018). Pioneering work by Manson *et al*. (2010) showed that the alkaloid gelsemine reduced the faecal intensity of *C. bombi* in *B. impatiens* workers. However, when *C. bombi* was directly exposed to gelsemine there was no significant reduction in parasite load. This suggests that a post-ingestive effect of the secondary metabolite may mediate the observed reduction in parasite load. Subsequent work has shown that the alkaloid nicotine reduced *C. bombi* parasite load in *B. terrestris* workers (Barrachi *et al*. 2015). Interestingly, again, direct exposure to nicotine had no effect on *C. bombi* parasite growth. Importantly in both of these investigations alkaloids were unable to fully clear

the *C. bombi* infection from the bumblebee host. However a reduction in infection intensity may be sufficient to reduce disease prevalence, due to the relationship between parasite reproduction and transmission (Ruiz-González & Brown 2006). Alkaloids are, however, only one class of bioactive secondary metabolite found in floral rewards. Flavonoids, diterpenoids and phenols are also present in floral rewards and have been shown to have bioactive properties (Cowan 1999, Adler 2000, Rojas *et al*. 2006, Li *et al*. 2013). In response to this diversity, work by Richardson *et al*. (2015) expanded our knowledge on the impact of phytochemicals on *C. bombi* infection. They investigated the effect of a range of plant metabolites, including alkaloids, terpenoids and iridoid glycosides, using *B. impatiens* and *C. bombi* as a model host-parasite system. Eight metabolites representative of compounds that *B. impatiens* is thought to naturally encounter and consume in the wild were investigated. Alkaloids again were shown to have the strongest effect on *C. bombi* parasite load, with anabasine having the strongest negative effect on *C. bombi* infection intensity. However, there is increasing evidence to suggest that different *C. bombi* strains have natural variation in phytochemical resistance (Palmer- Young *et al*. 2016). Consequently the effect of plant chemistry on bumblebee parasites should be tested, where possible, using a range of isolated parasite strains to ensure a consistent effect is recorded. While the mechanism of alkaloid mediated trypanosome reduction is yet to be identified, a number of suggestions have been proposed including interruption of parasite protein synthesis, interruption of metacyclogenesis (a trypanosome transitional phase) or simply by the upregulation of bumblebee immune function (Richardson *et al*. 2015). Given that bumblebees consistently consume a diverse suite of phytochemicals in their typical diet, it is also likely that some of these compounds may interact and have synergistic effects on diseases (Palmer-Young et al. 2017), although this largely remains to be explored.

As described above, our current knowledge on the impact of phytochemicals on bumblebee health has focused on interactions with the prevalent parasite *C. bombi*. Given that the microsporidian *N. bombi* may be more deleterious to bumblebee populations (Otti & Schmid-Hempel 2008, Cameron *et al*. 2011) I believe it is critical that the impact of phytochemicals on this host-parasite system be investigated as it may have greater implications for bumblebee health. For this to be the case, there need to be secondary phytochemicals that have antifungal properties, as microsporidia are now known to belong to the Fungi (Lee *et al*. 2008). For example, isoflavonoids, such

as biochanin A, have been isolated from red clover (*Trifolium pratense*) (Wu *et al*. 2003, Medjakovic & Jungbauer 2008), which is present in AES seed mixes. Biochanin A has been shown to have antifungal activity by competing for fungal cell wall receptor sites (Weidenbörner *et al*. 1990, Rojas *et al*. 2006) and also antiparasitic activity against promastigotes of *Leishmania chagasi* (Sartorelli *et al*. 2008). In addition a common constituent of pollen is the polyamide tricoumaroyl spermidine (Egan *et al*. 2018) and this phytochemical has known antifungal activity (Walters *et al*. 2001). These compounds are commonly encountered by foraging bumblebees in AES enhanced land and may provide a supplementary defense against microsporidian infection.

With the great diversity in pollen and nectar chemistry (Palmer-Young *et al*. 2018) it is likely that plants used in schemes that increase floral abundance and diversity, such as AES and CRP, may contain beneficial phytochemistry that indirectly improves pollinator health. Consequently, by identifying plants that bumblebees regularly come in to contact with in AES land, an overview of the chemical landscape can be mapped and any potential impacts on health can be investigated.

1.7 Conclusions

Some species of bumblebee are undergoing range and population declines. There is evidence to suggest that an increase in parasite prevalence and a reduction in wildflower meadows are two contributing factors. AES aim to mitigate the effect of intensive agriculture on insect pollinators by increasing floral abundance and diversity. It is evident that secondary metabolites encountered in floral rewards have negative fitness impacts for bumblebees. However, there is evidence to suggest that they may also have antimicrobial properties and may improve bumblebee health. To date this research has been limited to bumblebee-trypanosome host parasite interactions. A review of the pertinent literature has highlighted that bumblebees encounter a diverse suite of chemicals in their daily foraging routines. Investigating the floral chemistry of plants that bumblebees forage on and how this may indirectly impact bumblebee health would provide a deeper understanding of the ecological context of secondary metabolites in floral rewards and their impact on pollinators. Bumblebee larvae receive protein for growth directly from pollen. Consequently the chemical profiling of

commonly encountered pollens would enable investigation into any pharmacological effects of secondary metabolites on bumblebee brood diseases. Further to this a number of metabolites may have antifungal activity, and these have been reported in AES species. Consequently I recommend that investigating the impact of antifungal floral metabolites on the bumblebee (*B. terrestris*) and microsporidian (*N. bombi*) host-parasite system would provide a stimulating and novel route for investigation. The aim of the following five chapters is to address these recommendations. A brief summary of the aims and outcomes of each chapter is provided below.

Chapter two investigated whether bumblebee larvae are susceptible to infection with the trypanosome parasite *C. bombi*. In addition, larvae were monitored to see if they facilitated the transmission of *C. bombi* to naïve workers. This work enabled a deeper understanding of how bumblebee diseases interact with the developing brood.

Chapter three focused on identifying phytochemicals found in the pollen and nectar of plants used in Agri-environment schemes. Typifying the chemical composition of plants that bumblebees are known to forage on allowed insights into how the chemical landscape can impact bumblebee health. In addition four compounds with known biological activity were then used in an *in vitro* investigation to monitor any impact on the growth of *C. bombi*.

Chapter four investigated, for the first time, whether phytochemicals can impact on *N. bombi* infection *in vivo*. Infection with *N. bombi* may be more delerterious to bumblebees than other prevalent diseases and therefore this chapter provides the first evidence of how phytochemicals may impact microsporidian diseases in bumblebees. The therapeutic and prophylactic impact of the isoflavone biochanin A on both larvae and workers was explored.

Chapter five expanded on the proof of principle work in chapter four to investigate whether phytochemicals (caffeine & tricoumaroyl spermidine) found in AES plants, at their ecologically relevant concentrations, can impact on *N. bombi* infection *in vivo*.

Chapter six focused on the effect of caffeine on the epidemiology of *N. bombi* in wild caught and reared *B. terrestris* colonies. This chapter provides a holistic overview and

the first evidence of how phytochemicals can impact disease epidemiology in bumblebees.

Finally in the **discussion chapter** I discuss the implications of my findings and describe how they may influence future research into pollinator health.

Chapter 2

Larvae act as a transient transmission hub for the prevalent bumblebee parasite *Crithidia bombi*.

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Abstract

Disease transmission networks are key for understanding parasite epidemiology. Within the social insects, structured contact networks have been suggested to limit the spread of diseases to vulnerable members of their society, such as the queen or brood. However, even these complex social structures do not provide complete protection, as some diseases, which are transmitted by workers during brood care, can still infect the brood. Given the high rate of feeding interactions that occur in a social insect colony, larvae may act as disease transmission hubs. Here we use the bumblebee *Bombus terrestris* and its parasite *Crithidia bombi* to determine the role of brood in bumblebee disease transmission networks. Larvae that were artificially inoculated with *C. bombi* showed no signs of infection seven days after inoculation. However, larvae that received either an artificial inoculation or a contaminated feed from brood-caring workers were able to transmit the parasite to naive workers. These results suggest that the developing brood is a potential route of intracolonial disease transmission and should be included when considering social insect disease transmission networks.

Key words; *Bombus terrestris*, trypanosomatid, epidemiology, brood diseases

2.1 Introduction

To be successful, a parasite must have effective host transmission and the ability to maintain parasitaemia once infection is established (Price 1980). Understanding the epidemiology of parasites is consequently key for elucidating host-parasite interactions. The high population densities and low genetic variability of social insects may provide an ideal environment for pathogen transmission (Schmid-Hempel 1998, but see Van Baalen & Beekman 2006). Consequently, on top of individual immune mechanisms, some insect societies have evolved 'social immunity' (reviewed by Cremer *et al*. 2007). One potential mechanism of social immunity is the evolution or co-option of structured contact networks among individuals to minimize the spread of disease (Naug 2008, Naug & Smith 2007, Schmid-Hempel 1998, Schmid-Hempel & Schmid-Hempel 1993). Such networks, e.g., centrifugal polyethism in ants (Hölldober & Wilson 1990, Schmid-Hempel 1998) and heterogeneous interaction networks in honeybees (Naug 2008, Naug & Camazine 2002), may limit the spread of parasites within colonies, and give specific protection to the queen and brood, which are essential to the reproductive success of the colony. Nevertheless, even in large, complex insect societies, such protection is incomplete (Bailey 1956, reviewed by Bailey & Ball 1991, de Miranda & Genersch 2010, Hansen & Brødsgaard 1999). One reason for this is the high rate of worker-brood feeding interactions that are required for successful larval development. Consequently, in contrast to the prevailing view, brood may have the potential to act as a transmission hub in social insect colonies.

Bumblebees and their parasite, *Crithidia bombi*, provide an excellent model system to address this question. Bumblebees exist as small, relatively simple eusocial colonies (Wilson 1971). Most colonies acquire *Crithidia bombi* from transmission through foraging outside of the nest (Imhoof & Schmid-Hempel 1999, Jones & Brown 2014, Shykoff & Schmid-Hempel 1991), presumably from flowers that have been contaminated by a visiting infected worker (Durrer & Schmid-Hempel 1994, Ruiz-González *et al*. 2012). The parasite is transferred to other workers in the colony through contact networks (Otterstatter & Thomson 2007, Shykoff & Schmid-Hempel 1991). However, how these networks relate to the colony's brood is unclear. Currently, it remains unknown whether *C. bombi* is infective towards bumblebee larval stages, and thus whether bumblebee brood are integral to intra-colonial transmission networks. However, other insect trypanosomatids infect both adults and

larvae (e.g., Hamilton *et al*. 2015). In addition, even in the absence of infection, the repeated oral interactions (termed "trophallaxis", Wilson 1971) between larvae and adult workers could provide the opportunity for brood to act as a transient hub for parasite transmission. Here we ask the following questions: 1) can *B. terrestris* larvae become infected with the gut trypanosome *C. bombi*?, and 2) can larvae act as a source of infection for workers?

2.2 Materials and Methods

2.2.1 Colony origin

Four *B. terrestris* colonies (hereafter referred to as colonies A, B, C & D) (containing a queen, brood and a mean of 95 (± 6.1 S.E.) workers) were obtained from Biobest, Belgium. Colonies were kept in a dark room at 25ºC and 55% humidity (red light was used for colony manipulation). To ensure colonies were healthy and developing normally they were monitored for seven days prior to use in the experiments described below.

2.2.2 Testing whether bumblebee larvae can be infected by *Crithidia bombi*.

To create a parasite source, wild *B. terrestris* queens, naturally infected with *C. bombi*, were collected from Windsor Great Park, UK (SU992703), in the spring of 2016. The faeces of these bees were mixed with sugar water (1:1) and fed orally to ten workers removed from Colony D. The inoculated workers were returned to colony D, enabling parasite transmission to occur and creating a stock population for *C. bombi* acquisition. This stock population was maintained under dark room conditions (see above) and fed *ad libitum* pollen and sugar water.

A *C. bombi* inoculant was prepared by combining the faeces of twenty stock bees, which was then diluted in 1ml of 0.9% insect Ringer solution. To purify the *C. bombi* inoculant a modified version of the Cole (1970) triangulation protocol was used. The *C. bombi* cells in the resulting solution were counted using a Neubauer haemocytometer and the concentration of cells was calculated at 3,800/µl.

From the remaining three colonies (A, B & C) forty-two workers per colony were randomly selected and placed into individual quarantine. Quarantine chambers (n=126) comprised a 16×10×8cm plastic box. The lid was modified to allow the insertion of a 10ml tube to provide *ad libitum* sugar water, and each quarantine box was also provided with 0.1g of pollen. All quarantined bees were monitored for seven days. This period of time enabled reliable detection of already existing *C. bombi* infections (Logan *et al*. 2005, Schmid-Hempel & Schmid-Hempel 1993). All workers were then screened for the common parasites, *C. bombi, Nosema* spp. and *Apicystis bombi*, by microscopic examination of faecal samples using a phase contrast microscope at ×400 magnification. No infections were identified in any workers at this stage. These quarantined workers were used for all further experimental procedures to ensure there was no external parasite source.

Once workers had passed successfully through quarantine, ten larvae from each of the remaining three colonies were randomly assigned across experimental or control micro-colonies (1 each per colony). Each micro-colony (n=6) was housed in a 14×8×5.5 cm acrylic box and contained brood casing with five larvae at $2^{nd}/3^{rd}$ instar. Prior to inoculation these were kept without access to food or workers, under dark room conditions (outlined above) for one hour to ensure a feeding response. To inoculate larvae, sugar water and pollen were first combined (3:1) to create an artificial worker feed. This was then combined in equal proportions (100µl: 100µl) with the *C. bombi* inoculant (see above) to create a master-mix. Experimental larvae were exposed by opening the brood casing, and each was then fed 6µl of inoculated feed containing 11,400 parasite cells using a 20µl micropipette. Each control larva was fed 6µl of sugar water and pollen (3:1) in a similar fashion. Larvae were left to consume the entire inoculant until no trace was visible under a stereomicroscope (×20 magnification). Post inoculation the brood casing was resealed manually and larvae were returned to their micro-colonies. Each micro-colony was then given three quarantined workers to provide brood care, and was provisioned with *ad libitum* pollen and sugar water. After seven days, larvae were removed and their gut was isolated by dissection. The gut was homogenized in 0.5ml of 0.9% insect Ringer solution and screened for *C. bombi* by microscopic examination using a phase contrast microscope (×400 magnification). Workers from each micro-colony were also screened for *C. bombi* using microscopic examination of faecal samples. If an

infection was identified a Neubauer haemocytometer was used to calculate an average cell count.

2.2.3 Investigating whether larvae can act as a transmission hub for *Crithidia bombi*

Workers from each experimental micro-colony described above were found to have *C. bombi* infections. To investigate if this transmission had occurred during trophallaxis or via the inoculant residue left on the larvae a further experiment was designed. As before ten larvae were removed from each of the three donor commercial colonies (A, B & C). Experimental and control micro-colonies (n=6) each containing five larvae at $2^{nd}/3^{rd}$ instar were then set up. Larvae were inoculated as before, however once larvae had consumed the inoculum they were removed from their brood casing and submerged in 15 ml of ddH_2O and dried using a paper towel. Larvae were placed back in their brood casing which was resealed and returned to their respective micro-colonies with three quarantined workers to provide brood care, and were provisioned with *ad libitum* pollen and sugar water. After a period of seven days workers were removed and screened for *C. bombi* infection via microscopic examination of faeces. Again, under these conditions, workers were found to have *C. bombi* infections. A final, more conservative iteration of this methodology using the same sample size was undertaken where the cleaning process was repeated twice per larvae post inoculation. Workers were screened as before after a period of seven days.

As larval cleaning (see above) is not representative of an ecologically relevant scenario, to determine if larvae acted as a pathogen hub after receiving a parasite-contaminated feed, a serial transfer experiment was used. Again ten larvae were removed from each of the three donor commercial colonies (A, B & C). Experimental and control micro-colonies (n=6) each containing five larvae at $2^{nd}/3^{rd}$ instar were then set up. To ensure experimental manipulation was not the cause of *C. bombi* transmission, inoculation was undertaken by three workers per micro-colony (hereafter called “nurse cohort 0”), by providing them with a food source contaminated with *C. bombi* to feed to experimental larvae. A *C. bombi* inoculant, as described above, was mixed with artificial worker feed in a 1:5 ratio (to make up 1ml), which nurse cohort 0 were allowed to feed to experimental larvae for a period

of 24 hours. Control groups were similarly fed a pollen sugar water mix. At the end of the 24-hour period the brood casing containing all larvae was removed and placed into a sterile micro-colony box with three newly quarantined workers (hereafter called "nurse cohort 1") and provided *ad libitum* pollen and sugar water. After 24 hours exposure nurse cohort 1 were removed and quarantined for seven days before screening for *C. bombi* (as described earlier). Larvae were simultaneously transferred into a new sterile micro-colony box and provisioned with three newly quarantined workers (hereafter called "nurse cohort 2") and *ad libitum* pollen and sugar water. This serial transfer continued for a total of three days post inoculation, with all workers being screened for *C. bombi* infections seven days after exposure to the contaminated larvae. If infection was observed a haemocytometer was used to quantify the parasite load. In all experiments infection intensities were compared with ANOVA tests using R programming language (R Core Team 2016). All graphical outputs were undertaken in R, using ggplot 2 (Wickham 2009).

2.3 Results

2.3.1 Can *Crithidia bombi* infect *Bombus terrestris* larvae?

All *B. terrestris* larvae (n=15) across three micro-colonies showed no signs of *C. bombi* infection seven days after inoculation. However transmission of the parasite from the larvae to the workers occurred in all artificially inoculated micro-colonies with 100% of workers becoming infected. There were no statistical differences in worker infection intensity across the micro-colonies ($F_{2,6} = 3.35,\ P = 0.1$). Neither larvae nor workers from the control replicates showed any sign of infection, as expected.

2.3.2 Can larvae act as a transmission hub for *Crithidia bombi*?

In the first experiment, where larvae were washed once after experimental inoculation, all nine workers that were exposed to these larvae became infected by *C. bombi.* There was no statistical difference across micro-colonies in the infection

intensities of workers after larval washing ($F_{2,6}$ =1.82, P = 0.24). None of the workers in the control colonies became infected, as expected.

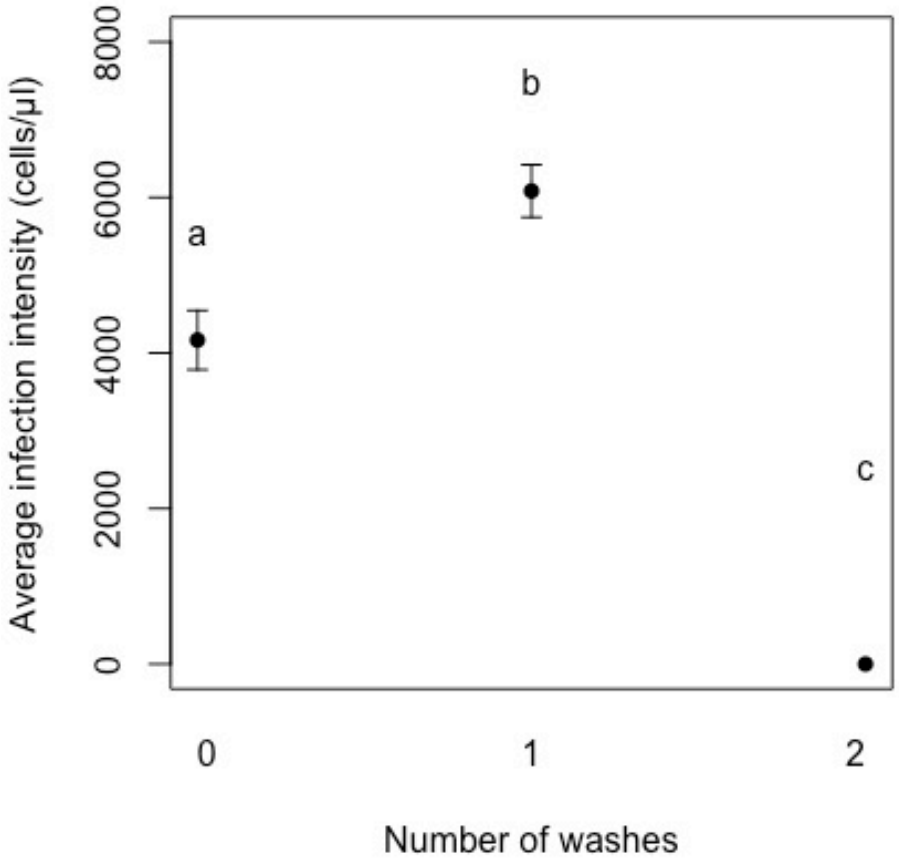

Figure 2. 1 *C. bombi* infection intensity in *B. terrestris* workers (mean ± SEM) seven days after exposure to artificially inoculated larvae that underwent different washing regimes. X-axis denotes number of experimental washes. Statistical differences between treatments are represented with letters ($F_{2,26}$=111.44, P=<0.001).

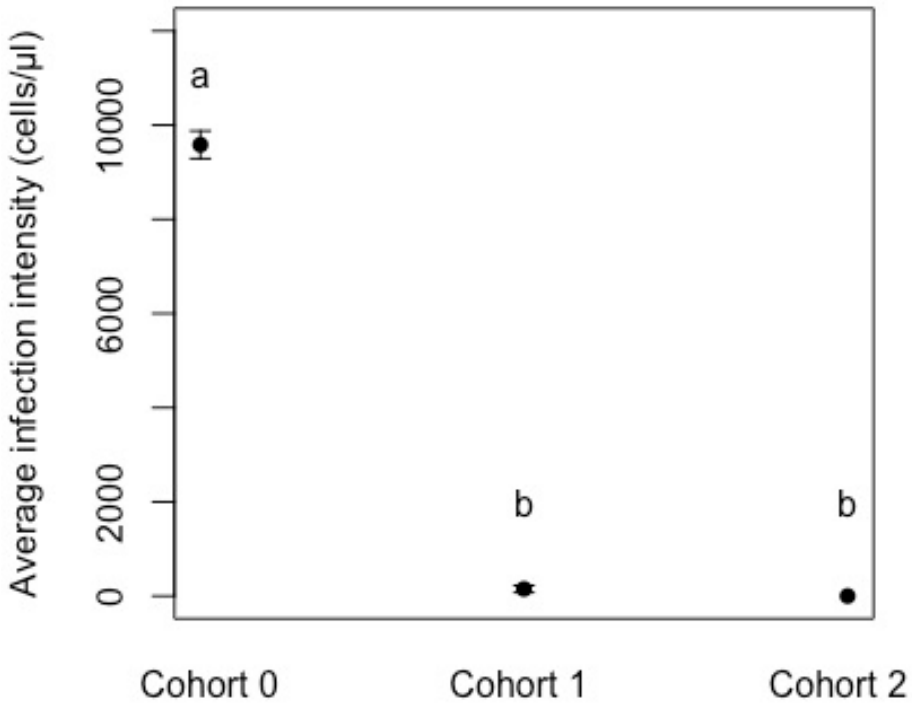

Figure 2. 2 *C. bombi* infection intensity in *B. terrestris* workers from three nurse cohorts of the serial transfer experiment (mean ± SEM) seven days after exposure to inoculated larvae. Statistical differences between nurse cohorts are represented with letters ($F_{2,26}$*=977.57, P=<0.001*).

In the second experiment, where larvae were washed twice after inoculation, no exposed workers developed a *C. bombi* infection. Including the first experiment with no larval cleaning, infection intensities were significantly different across all three washing treatments ($F_{2,26}$*=111.44, P=<0.001*) (Figure 1).

In the serial-transfer experiment, nurse cohort 0 that were used to inoculate larvae prior to the serial transfer of brood were quarantined for seven days before being screened, and all tested positive for *C. bombi* infection. Again, infection intensities did not differ significantly among micro-colonies ($F_{2,6}$*= 3.81, P = 0.09*). These workers had come into direct contact with the *C. bombi* inoculant while feeding larvae, and screening them confirmed that the inoculum was infective to workers. A

third of quarantined bees (n=9) from the nurse cohort 1 developed *C. bombi* infections, although infection intensities were 100-fold lower compared to nurse cohort 0, and no infections were identified in bees from nurse cohort 2. Due to the number of bees infected after the first serial transfer, it was not possible to analyze infection intensity across micro-colonies. However, infection intensities in nurse cohort 1 and 2 were significantly lower than in nurse cohort 0 ($F_{2,26}=977.57$, $P=<0.001$)(Figure 2).

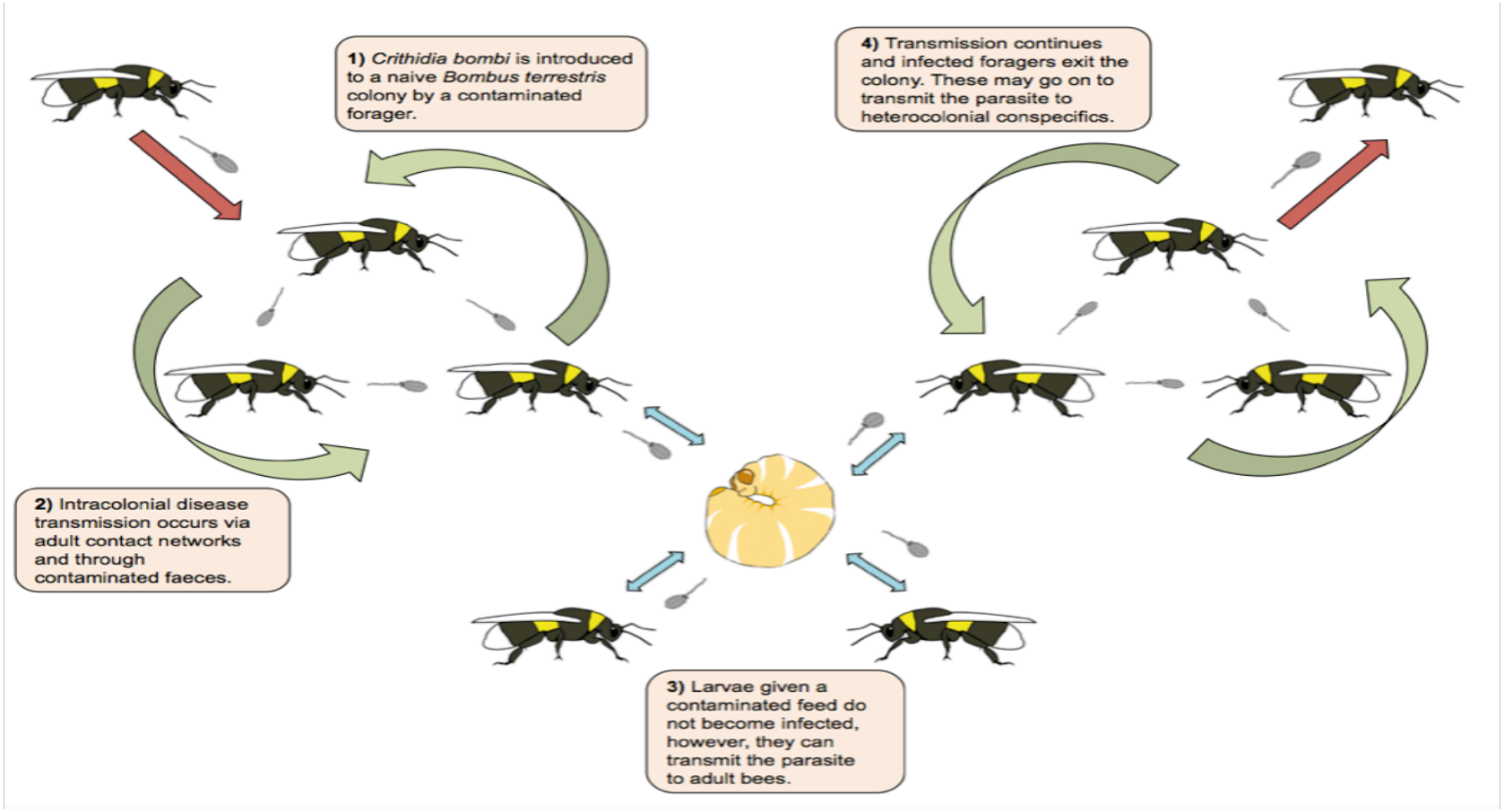

Figure 2.3 Intracolonial transmission of *Crithidia bombi*, which includes the developing brood as a potential route of microbial disease transmission in *Bombus terrestris.*

2.4 Discussion

Here we have shown that under laboratory conditions *B. terrestris* larvae had no signs of *C. bombi* infection seven days after inoculation. However, the feeding interaction between workers and larvae facilitated the transmission of *C. bombi*, with the larvae acting as a transient parasite transmission hub.

Microbial parasites and their invertebrate hosts have been co-evolving in an ecological arms race to either maintain parasitaemia or to reduce parasite loads respectively (Anderson & May 1981, Cremer *et al*. 2007, Price 1980, Schmid-Hempel 1998). Given that other insect trypanosomatids can infect both adults and larvae (Hamilton 2015), and given the likely frequent contact between bumblebee larvae and *C. bombi*, it is surprising that the parasite has not evolved to exploit these additional hosts. However, in high sugar, aqueous environments the ability of *C. bombi* to persist is drastically reduced (Cisarovsky & Schmid-Hempel 2014) and this may explain the inability of *C. bombi* to infect bumblebee larvae. Bumblebee larval guts are likely to have high osmotic potential due to the sugar heavy feeding regime they receive during broodcare (Pereboom 2000). Whilst larvae must consume and metabolize sugars for growth and development they do not defecate and therefore it is likely that the high osmotic potential in their gut undergoes less reduction than might be expected in adult workers which regularly defecate. Differences in gut conditions between the two bumblebee life stages may therefore explain why only adults support *C. bombi* colonization and subsequent infection.

Previous work has highlighted that contact networks between adults are a significant predictor of *C. bombi* infection risk (Otterstatter & Thomson 2007). Our results suggest that larvae should also be integrated into bumblebee disease contact networks. In adult bumblebees *C. bombi* transmission rate is high as the infection is spread via contaminated faeces (Schmid-Hempel 1998). Our results, combined with the high frequency of feeding interactions that occur in a bumblebee nest (Katayama 1973), suggest that the parasite is able to indirectly use larvae as a transmission hub to infect naive adult hosts. At a more general level, bumblebees are not noted for their hygienic nest conditions (Wilson 1971) and therefore trophallactic interactions between workers and larvae are likely to be contaminated with an array of parasites. Consequently, larval transmission hubs could be relevant for other bumblebee parasites that are dependent on contact networks (Fürst *et al*. 2014, Rutrecht & Brown

2008), particularly given the large proportion of the colony workforce that is involved in brood care (Pendrel & Plowright 1981). Thus, we suggest that adult-brood trophallactic transmission is likely to be a component of social insect disease networks generally.

When larvae received either zero or one wash post inoculation, workers providing brood care went on to develop *C. bombi* infections that are consistent with previously reported infection intensities (Logan *et al*. 2005, Schmid-Hempel & Schmid-Hempel 1993). Interestingly, when larvae were washed twice post inoculation, brood caring workers did not become infected with *C. bombi*. The rationale behind larval cleaning was to provide evidence that transmission of parasites occurred during trophallaxis and not from inoculant residue left on the larvae. Our data show a non-linear response across washing treatments. One explanation for this is that submerging larvae in ddH_2O would be an unnatural stress that may cause larvae to open their buccal cavity, consequently washing out any residual parasites. It is likely that two washes effectively removed all parasite cells from both the larval surface and the buccal cavity. In contrast, in the one wash treatment, our worker infection results show that not all parasite cells were removed.

Following inoculation of larvae by nurse cohort 0, disease establishment only occurred in nurse cohort 1 in the serial transfer experiment, demonstrating the transient nature of transmission via brood-worker interactions. However, we would note that, under natural conditions, brood are likely being repeatedly exposed to the parasite through feeding interactions, and thus whilst transmission is transient after a single feed, it is likely to be continuous under natural conditions. We acknowledge that bumblebee parasite transmission may occur on surfaces (Durrer & Schmid-Hempel 1994, Ruiz-González *et al*. 2012) and that brood casing may facilitate this. However, brood-caring workers are primarily focused on direct contact with developing larvae and this would be the exchange point for contaminated food. In addition, experimentally cleaned larvae were able to transmit the parasite to naïve workers suggesting that disease transmission is likely to be occurring during trophallaxis, rather than through contact with brood casing. Nurse cohort 0, which were exposed directly (through feeding the larvae) to the parasite source during the serial transfer experiment went on to develop *C. bombi* infections that are consistent with previously reported infection intensities (Logan *et al*. 2005, Schmid-Hempel & Schmid-Hempel 1993). Parasitaemia in workers infected during the experimental

cleaning methodology was also consistent with previously reported infection intensities. Interestingly however, even after an incubation period of seven days the parasite loads of infected workers from nurse cohort 1 were a 100-fold lower than what has been reported in *C. bombi* parasitaemia (Logan *et al*. 2005). Previous work has shown that a dose of 5,000 cells is enough to lead to infection (Brown *et al*. 2003), but how dose relates to subsequent infection intensity remains unknown. One explanation for the low infection intensities in the serial transfer experiment may therefore be that they were initiated by significantly fewer parasite cells. It seems unlikely that these lower parasitaemias are due to differences in individual susceptibility (Barribeau & Schmid-Hempel 2013, Brunner *et al*. 2013, Sadd & Barribeau 2013) particularly as the workers that exhibited low infection intensities were a random sample from the same colonies as the first round of workers that showed much higher infection intensities.

Bumblebee parasite prevalence is increasing (Cameron *et al*. 2016, Schmid-Hempel *et al*. 2014) and this has been linked to bumblebee population declines (Cameron *et al*. 2011). More specifically, the global prevalence of *C. bombi* is rising and this has been linked to the transportation of commercial bumblebee colonies for pollination services (Graystock *et al*. 2013, Whitehorn *et al*. 2013). *C. bombi* has significant impacts on queen fitness (Brown *et al*. 2003), high transmission potential (Otterstatter & Thomson 2007, Schmid-Hempel & Schmid-Hempel 1993), and high prevalence (Jones & Brown 2014, Shykoff & Schmid-Hempel 1991). Here we have identified that bumblebee larvae can act as an intracolonial transmission hub for *C. bombi*. We believe that these results enhance our understanding of the transmission potential of *C. bombi* and help to explain how it maintains such a high environmental prevalence.

Chapter 3

The floral chemistry of Agri-environment schemes and its effect on bumblebee parasites

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N.B –*Tricoumaroyl spermidine synthesis was carried out by Arran J. Folly and Iain W. Farell*

Abstract

Agri-environment schemes (AES) are used across Europe to enhance agricultural landscapes and to support biodiversity. Contained within such schemes are prescriptions that aim to support insect pollinators by increasing the abundance and diversity of flowering plants. These prescriptions have been shown in turn to increase pollinator abundance and diversity. However, pollen and nectar, the main attractants for insect pollinators, contain secondary metabolites. These phytochemicals can have negative impacts on pollinators, but some may have antimicrobial activity and may therefore indirectly improve pollinator health. Here, using high performance liquid chromatography and mass spectrometry I screened the pollen and nectar from nine flowering plant species included in UK based AES, on which bumblebees are known to forage. I then tested four of these compounds *in vitro* against two isolated strains of the important bumblebee parasite *Crithidia bombi* to test for biological activity. Caffeine, a methylxanthine identified in the nectar of *Onobrychis viciifolia* (Sainfoin), increases *C. bombi* growth at my recovered ecologically relevant concentration, before having an inhibitory effect at higher, yet still ecologically relevant concentrations. These results suggest that a single phytochemical may have conflicting impacts on bumblebee health depending on the concentration at which it is encountered, and consequently determining the chemical profile of key forage plants is critical in understanding how florally enriched landscapes impact on pollinator health.

Key words; Trypanosomatid, *Crithidia bombi*, *Bombus terrestris*, caffeine, secondary metabolite, phytochemical, liquid chromatography, mass spectrometry, *in vitro*

3.1 Introduction

Following the Second World War and a requirement to increase national food production, intensive agriculture is now the dominant land use in Western Europe (Robinson & Sutherland 2002). However, the intensification of agriculture has led to fewer assemblages of natural, heterogeneous habitats and associated biodiversity (Krebs *et al*. 1999, Donald *et al*. 2001). More specifically, a decline in wild flower meadows has resulted in a reduction in both insect pollinator populations and diversity (Carvel *et al*. 2006, Biesmeijer *et al*. 2006, Potts *et al*. 2010, Ollerton *et al*. 2014). In an attempt to mitigate the effect of intensive agriculture on wild animal populations, Agri-environment schemes (AES) have been developed (EU Common Agricultural Policy 2015). Currently 25% of the European Union's utilized agricultural area comes under AES and the expenditure on such schemes for 2007-2013 was €2.3 Billion (Science for Environment Policy 2017). However these schemes are more prominent in England with approximately 70% of agricultural land currently under AES (DEFRA 2015). Within AES, prescriptions are set out that aim to increase both floral abundance and diversity to benefit pollinators (EU Common Agricultural Policy 2015, Natural England 2017). Previous AES policy development has interacted with the scientific community on how such strategies can be enhanced for insect pollinators (Pywell *et al*. 2011 (a), Dicks *et al*. 2015) and there is a growing body of evidence that show these strategies are increasing insect pollinator abundance (Pywell *et al*. 2006, Carvell *et al*. 2007, Wood *et al*. 2015, Carvell *et al*. 2017). Interestingly however, floral rewards, the main attractant for insect pollinators, contain secondary metabolites (Baker & Baker 1975, Adler 2000, Stevenson *et al*. 2017). These phytochemicals can have negative impacts on pollinators (Cook *et al*. 2013, Tiedeken *et al*. 2016). However some phytochemicals may have antimicrobial properties and may therefore have positive effects by reducing parasite loads (Manson *et al*. 2010). Consequently, strategies that increase floral abundance such as AES may indirectly impact pollinator health.

Economically important pollinators such as bees (Breeze *et al*. 2011) are entirely dependent on pollen and nectar as a source of protein and carbohydrates, respectively (Wilson 1971). In addition, floral rewards also provide a suite of essential amino acids and vitamins (Auclair & Jamieson 1948). These constituents are essential for bee nutrition, but perhaps more notable is the requirement for a

polyfloral diet (Alaux *et al*. 2010) that may provide protection against microbial parasites (Pasquale *et al*. 2013). Bumblebees are primitively eusocial pollinators that are exposed to a diverse network of parasites that can impact upon their health (Schmid-Hempel 1998). One well-studied example is the gut trypanosome *Crithidia bombi* (Gorbunov 1987, Schmid-Hempel & Schmid-Hempel 1993). This internal parasite has high environmental prevalence (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014) and is transmitted horizontally presumably via flowers (Durrer & Schmid-Hempel 1994, Ruiz-Gonzalez *et al.* 2012, Adler *et al*. 2018) and adult contact networks (Otterstatter & Thomson 2007) or by shared contact with larvae (Folly *et al*. 2017). Infection with *C. bombi* can reduce colony founding success and bumblebee colony fitness by up to 40% (Brown *et al*. 2003). Previous work has shown that secondary metabolites found in pollen and nectar can affect *C. bombi* development *in vivo* (Manson *et al*. 2010, Richardson *et al*. 2015) and more recently this relationship has been identified *in vitro* (Palmer-Young *et al*. 2016, Palmer-Young & Thursfield 2017). The ability to isolate and culture *C. bombi* under controlled conditions (Salathé *et al*. 2012) makes it an ideal study system to investigate whether the diverse floral biochemistry, produced by initiatives such as AES, may have an impact on bumblebee health. However, in some instances the reported antimicrobial activity of phytochemicals on *C. bombi* has been identified when testing concentrations above their ecologically relevant values (Palmer-Young *et al*. 2017) and these findings, whilst useful to identify new areas of research or to test methodologies, can be misleading for policy makers on how best to formulate planting strategies that may benefit insect pollinators.

To date the floral chemistry of plants recommended for use in AES has remained un-investigated despite the obvious implications for pollinator health. Here we screen flowers recommended in AES, on which bumblebees are known to forage, for phytochemicals in both the pollen and nectar using a combination of high performance liquid chromatography and mass spectrometry (LC-MS). We then identify phytochemicals that have reported biological activity and test them *in vitro* at ecologically relevant concentrations on *C. bombi* to investigate whether they may indirectly impact bumblebee health.

3.2 Methods

3.2.1 Identification and analysis of phytochemicals from AES plants

Target flowers were identified from recommended AES seed mixes prescribed by Natural England (2017) that bumblebees are known to forage on (Table 3.1). Fresh pollen and nectar was collected from Langridge, UK (ST741694), Salisbury Plains, UK (SU069440), Royal Botanic Gardens Kew (RBG Kew), UK (TQ184769) and Egham, UK (TQ010709) between May 2016 and July 2017. This collection period enabled repeat sampling of species and also allowed for sampling of any species that may have already undergone anthesis. Flowers were initially covered using a muslin cloth and a cable tie for up to 24 hours. This ensured maximum harvest of both pollen and nectar (Corbet 2003). Nectar was collected using a 2µl glass micro-capillary tube (Morrant *et al*. 2009) and stored in a 1.5 ml UV protective Eppendorf tube. Pollen was removed by positioning a stamen over a separate 1.5 ml UV protective Eppendorf tube and rocking the target flower backwards and forwards. Both pollen and nectar samples were kept in a chilled container prior to being stored in a -20°C freezer for subsequent analysis. For each species of plant, nectar and pollen samples were pooled separately, this enabled the detection of phytochemicals from different locations within the UK. In addition, pooling samples from multiple individuals of the same species increased the likelihood of detecting phytochemicals that are found at naturally low concentrations within both pollen and nectar.

Chemical analysis of floral rewards was undertaken using LC-MS. Each pollen sample was initially centrifuged at 6000 rpm for 1 minute before 100µl of 100% methanol (MeOH) was added. Pollen samples were then vortexed for 20 seconds prior to being stored at room temperature for 24 hours to ensure maximum extraction of phytochemicals into the methanol solvent. Nectar samples were centrifuged at 6000 rpm for 1 minute before 50µl of 100% MeOH was added and were available for immediate analysis after 20 seconds of vortexing.

Once prepared, as outlined above, a 50µl aliquot of each sample was placed into an individually labeled low absorption LC vial. LC-MS analysis was carried out using an LC elution program described in Tiedeken *et al*. (2014) on a Thermoscientific Ultimate 3000 LC and Velos Pro MS detector, with 5µl injection volume onto a Phenomenex Luna C18 (2) column (150 x 4.0mm id, 5µm particle

size) held at 30°C. A gradient elution was employed consisting of a mobile phase of (A) MeOH (B) H_20 and (C) 1% HCO_2H in MeCN at a flow rate of 0.5mL min^{-1}. Phytochemicals were then identified on the resulting ion chromatogram in Xcalibur 2.1.0 (2012) using retention times, molecular weight and UV absorption as proxies for identification. Phytochemicals that could not be identified from these parameters using the RBG Kew phytochemical library were subjected to High Resolution Electrospray Ionization Mass Spectrometry (HR-ESI-MS) using a Thermoscientific LTQ Orbitrap^{tm} XL with a 5µl injection volume onto a Phenomenex Luna C18 (150mm x 3mm id, 3µm particle size) held at 30°C. A gradient elution was employed consisting of a mobile phase of (A) H_2O (B) MeOH (C) 1% CH_2O_2 in CH_3CN at a flow rate of 0.4mL min-1. HR-ESI-MS analysis facilitated identification of phytochemicals from fragmentation patterns of the mass spectra and accurate molecular formulae.

All identified phytochemicals (in pollen = Table 3.2 & in nectar = Table 3.3) were then assigned a concentration in parts per million (ppm) by comparison of UV peak areas with standard curves at relevant floral reward concentrations (10, 100, 500, 1000 ppm) (Adler 2000, Cook *et al*. 2013). In the absence of a specific standard, compounds with a similar UV absorption, based on the same chromophore, were used as an approximation.

By conducting a targeted literature search each phytochemical was investigated to identify any biological activity that may have an effect on the proposed study system. The alkaloid caffeine at 39ppm (Sigma Aldrich CO750) recovered from the nectar of *Onobrychis viciifolia* (sainfoin), the plant hormone abscisic acid at 125ppm (Sigma Aldrich 862169) recovered from the nectar of *Malva moschata* (musk mallow), the polyamide tricoumaroyl spermidine (TCS) at 208ppm (produced via synthesis (Appendix 9.1)) recovered from the pollen of *Lotus corniculatus* (bird's-foot trefoil) and the isoflavone biochanin A at 0.1ppm (Sigma Aldrich, D2016) recovered from the nectar of *Trifolium pratense* (red clover), were selected to be used in the investigation as these have reported biological activity (Raj & Dhalla 1965 (caffeine), Negri *et al.* 2015 (abscisic acid), Walters *et al*. 2001 (TCS) and Weidenbörner *et al*. 1990 (biochanin A)) and were available in sufficient quantities for an *in vitro* investigation.

Table 3.1 Flowers which are visited by bumblebees, from seed mixes, outlined by Natural England 2017, and that are recommended for use in UK based Agri-environment schemes. Other plants contained in the mixes, not listed or tested here, are selected for birds, hoverflies and other nectarivorous animals.

Species	Family	Seed mix	Annual/ Perennial	Peak flowering time
Centuarea nigra	Asteraceae	Bumblebird (AB16), Nectar Flower seed mix (AB1), Flower rich margins + plots (AB8)	Perennial	June-September
Lotus corniculatus	Fabaceae	Bumblebird (AB16), Nectar Flower seed mix (AB1), Flower rich margins + plots (AB8)	Perennial	June-August
Malva moschata	Malvaceae	Nectar Flower seed mix (AB1)	Perennial	June-August
Onobrychis viciifolia	Fabaceae	Nectar Flower seed mix (AB1)	Perennial	June-August
Prunella vulgaris	Lamiaceae	Flower rich margins + plots (AB8)	Perennial	June-September
Ranunculus acris	Ranunculaceae	Flower rich margins + plots (AB8)	Perennial	April-October
Rhinanthus minor	Orobanchaceae	Flower rich margins + plots (AB8)	Annual	May-September
Trifolium spp.	Fabaceae	Nectar Flower seed mix (AB1)	Perennial	May-September
Vicia sativa	Fabaceae	Bumblebird (AB16)	Annual	May-September

3.2.2 Testing the phytochemical activity of AES plants on *Crithidia bombi in vitro*

Two parasite strains were isolated and cultured from *Bombus terrestris* queens found naturally infected with *C. bombi* at RBG Kew in 2017, using a methodology adapted from Salathé *et al*. (2012). Once isolated, strains were individually propagated once per week by transferring 200µl of the developing parasite medium into 6ml of fresh culture medium before being stored in a CO_2 incubator, set to 27°C with a 3% CO_2 concentration. This weekly propagation continued for the duration of the experiment.

To create a working stock solution, each test compound was weighed using an electronic balance and diluted into 100µl of 40% MeOH. This solution was then diluted to the required test concentration by adding 10ml of double distilled H_2O (DDH_2O), which also reduced the MeOH concentration to 0.004%. For each

compound two 2ml deep, 96 well spectrophotometer plates with covers were set up, one for each parasite strain, in a dedicated, sterile laminar flow hood. To minimize edge effects from spectrophotometer reads an outer perimeter of 200µl DDH_2O per well was used. Each column of the plate represented a different treatment and each row within a column was a replicate of the designated treatment. Each phytochemical was tested at its ecologically relevant concentration and to create a growth curve each phytochemical was also tested at x2 and x5 its ecologically relevant concentration. To achieve this 100µl of parasite culture containing 100 cells/µl was reverse pipetted into a target well using a sterile filter tip. Then using a sterile filter 100µl of the phytochemical at the required concentration dissolved in DDH_2O was added. In addition to these three treatments a negative control treatment, using 100µl of DDH_2O and 100µl of culture medium, and a positive control, using 100µl of cultured parasites (100 cells/µl) and 100µl of DDH_2O, were also included in each plate.

Once plates had been established they were immediately read using a Tecan Infinite M200 plate reader, with a 30°C incubation temperature and a 15 second orbital shake with 2mm amplitude before reads started to resuspend parasite cells. Each well was subject to 4x4 reads at 630nm with a 500ms shake before each read. Once plates had been read they were returned to the dedicated CO_2 incubator and then read using the same parameters every 24 hours for a total period of 7 days. The resulting dataset was visualized in I-control^{tm} version 1.5. At the end of the 7-day timeframe 15µl was removed from each experimental well to take a direct parasite count using a Neubauer improved haemocytometer on a phase contrast microscope at ×400 magnification.

3.2.3 Statistical analysis

All statistical analyses were undertaken in R open source programming language (R Core Team 2017). To analyse the effect of each phytochemical on *C. bombi* optical density and final parasite count, two separate linear mixed effects models (LMM) were used. Models were constructed in the R package 'lme4' (Bates *et al*. 2015). Firstly, optical density was used as a response variable, with phytochemical treatment and 24-hour period as designated covariates. The model also incorporated parasite strain as a random effect. In the second model final parasite count data was selected as a response variable with phytochemical treatment as a covariate. Because the final

parasite count data took place at single time point I removed 24-hour period as a covariate. As before parasite strain was included as a random effect. Models were validated in R by visually checking the normality of residuals, and for overdispersion and collinearity of variables.

Table 3.2 Phytochemicals recovered using LC-MS from the pollen of plants in UK agri-environment schemes.

Species	Phytochemical Identification	RT (30 min)	M/Z	Ion (I)	Formula (I)	Formula	MW	PPM	Phytochemical type
Centaurea nigra	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	13.82	787.3707	[M+H]+	$C_{46}H_{51}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	122	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	14.05	787.3698	[M+H]+	$C_{46}H_{51}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	122	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	14.27	787.3699	[M+H]+	$C_{46}H_{51}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	122	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	14.47	787.3698	[M+H]+	$C_{46}H_{51}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	122	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	14.75	787.3706	[M+H]+	$C_{46}H_{51}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	122	Amide
	Unknown	13.01	710.3172	[M+NH4]+	$C_{38}H_{48}NO_{12}$	$C_{38}H_{44}O_{12}$	692	31	Diterpene
	Unknown	13.01	693.29102	[M+H]+	$C_{39}H_{41}N_4O_8$	$C_{39}H_{40}N_4O_8$	692	31	Diterpene
Lotus corniculatus	6-Methoxykaempferol 3-*O*-dihexose	8.73	641.77	[M+H]+	$C_{28}H_{33}O_{17}$	$C_{28}H_{32}O_{17}$	N/A	N/A	Flavonoid
	N,N,N- Tricoumaroylspermidine	13.85	584.2755	[M+H]+	$C_{34}H_{38}N_3O_6$	$C_{34}H_{37}N_3O_6$	583	208	Polyamide
	Unknown	14.25	309.1234	[M+H]+	$C_{18}H_{17}N_2O_3$	$C_{18}H_{16}N_2O_3$	308	81	Alkaloid
	Iristectorigenin A	14.85	331.0812	[M+H]+	$C_{17}H_{15}O_7$	$C_{17}H_{14}O_7$	330	N/A	Flavonoid
	Unknown	16.21	345.0963	[M+H]+	$C_{18}H_{17}O_7$	$C_{18}H_{16}O_7$	344	N/A	Flavonoid
Malva moschata	Adenosine	2.54	268.1	[M+H]+	$C_{10}H_{14}N_5O_4$	$C_{10}H_{13}N_5O_4$	267	17	Purine nucleoside
	Unknown	3.06	208.06	[M+H]+	$C_{10}H_{10}N0_4$	$C_{10}H_9N0_4$	207	N/A	Aecetic acid
	Tryptophan	4.6		[M+H]+	$C_{11}H_{13}N_2O_2$	$C_{11}H_{12}N_2O_2$	204	41	Amino acid
	3,3'4',5,7-Pentahydroxy-6-methoxyflavone, 3-O-[β-D-Glucopyranosyl (1-6) β-D-Glucopyranosyl]	7.93	657.1647	[M+H]+	$C_{28}H_{33}O_{18}$	$C_{28}H_{32}O_{18}$	656	96	Flavonoid
	3,4',5,6,7- Pentahydroxyflavone, 6,7-Methylene ether, 3-O-[β-D-Glucopyranosyl (1-6) β-D-glucopyranosyl] m Patuletin	8.73	801.2087	[M+H]+	$C_{34}H_{41}O_{22}$	$C_{34}H_{40}O_{22}$	800	79	Flavonoid
	6-Methoxykaempferol-3-*O*-[rhamnosyl-hexoside]	9.36	625.1759	[M+H]+	$C_{28}H_{33}O_{16}$	$C_{28}H_{32}O_{16}$	624	208	Flavonoid

	6-Methoxykaempferol-3-*O*-[rhamnosyl-di-hexoside]	9.36	787.22955	[M+H]+	$C_{34}H_{43}O_{21}$	$C_{34}H_{42}O_{21}$	786	208	Flavonoid
	Isorhamnetin-3-*O*-[α-L-Rhamnopyranosyl-(1→2)-[3S-hydroxy-3-methylglutaroyl-(→6)]-β-D-glucopyranoside]	10.42	769.2181	[M+H]+	$C_{34}H_{41}O_{20}$	$C_{34}H_{40}O_{20}$	768	102	Flavonoid
	Isorhamnetin 3-*O*-rhamnosyl-6 (rhamnosyl-2)-glucosyl	12.41	771.2134	[M+H]+	$C_{37}H_{39}O_{18}$	$C_{37}H_{38}O_{18}$	770	118	Flavonoid
Onobrychis viciifolia	Unknown	11.63	679.511	[M+H]+	$C_{40}H_{71}O_8$	$C_{40}H_{70}O_8$	678	17	Diterpene
	Unknown	12.46	679.5117	[M+H]+	$C_{40}H_{71}O_8$	$C_{40}H_{70}O_8$	678	19	Diterpene
	Unknown	18.47	548.34235	[M+NH4]+	$C_{27}H_{50}NO_{10}$	$C_{27}H_{46}O_{10}$	530	0.7	Diterpene
Prunella vulgaris	No metabolites identified								
Ranunculus acris	Sophoraflavonoloside	8.55	611.1608	[M+H]+	$C_{27}H_{31}O_{16}$	$C_{27}H_{30}O_{16}$	610	50	Flavonoid
Rhinanthus minor	Isorhamnetin 3-*O*-rutinoside, 7-*O*-hexoside	8.66	787.2297	[M+H]+	$C_{34}H_{43}O_{21}$	$C_{34}H_{42}O_{21}$	786	N/A	Flavonoid
Trifolium pratense	Isorhamnetin neohesperidoside	8.63	641.17023	[M+H]+	$C_{28}H_{33}O_{17}$	$C_{28}H_{32}O_{17}$	640	8	Flavonoid
	Triscaffeoyl spermidine E,E,Z	10.94	632.25867	[M+H]+	$C_{34}H_{38}N_3O_9$	$C_{34}H_{37}N_3O_9$	631	43	Amide
	Triscaffeoyl spermidine E,Z,Z	11.37	632.25916	[M+H]+	$C_{34}H_{38}N_3O_9$	$C_{34}H_{37}N_3O_9$	631	43	Amide
	Triscaffeoyl spermidine, E,E,E	11.79	632.25836	[M+H]+	$C_{34}H_{38}N_3O_9$	$C_{34}H_{37}N_3O_9$	631	43	Amide
	Trisferuloyl spermidine, E,E,E	14.16	674.30597	[M+H]+	$C_{37}H_{44}N_3O_9$	$C_{37}H_{43}N_3O_9$	673	43	Amide
	Feruloyl, dicaffeoyl spermidine	12.67	646.27478	[M+H]+	$C_{35}H_{40}N_3O_9$	$C_{35}H_{39}N_3O_9$	645	31	Amide
	Feruloyl, dicaffeoyl spermidine	12.51	646.27527	[M+H]+	$C_{35}H_{40}N_3O_9$	$C_{35}H_{39}N_3O_9$	645	31	Amide
	Quercetin-3-*O*-(6-*O*-malonylgalactoside)	10.87	551.10217	[M+H]+	$C_{24}H_{23}O_{15}$	$C_{24}H_{22}O_{15}$	550	0.1	Flavonoid

	Biochanin A	17.46	285.07556	[M+H]+	$C_{16}H_{13}O_5$	$C_{16}H_{12}O_5$	284	0.1	Flavonoid
	Biochanin A 7-*O*-(6'-*O*-malonylglucoside)	14.96	533.12805	[M+H]+	$C_{25}H_{25}O_{13}$	$C_{25}H_{24}O_{13}$	532	0.1	Amide
	Sissotrin (Biochanin A glucoside)	13.86	447.1282	[M+H]+	$C_{22}H_{23}O_{10}$	$C_{22}H_{22}O_{10}$	446	0.6	Amide
	Kaempferol-3-*O*-(6'-*O*-malonyl-hexoside)	11.78	535.10773	[M+H]+	$C_{24}H_{23}O_{14}$	$C_{24}H_{22}O_{14}$	534	0.08	Flavonoid
Vicia sativa	N-Jasonoyltryptophan	11.19	397.18	[M+H]+	$C_{23}H_{29}N_2O_4$	$C_{23}H_{28}N_2O_4$	396	32	Amino acid
	Dicoumaroyl putrescene	12.12	381.18	[M+H]+	$C_{22}H_{25}N_2O_4$	$C_{22}H_{24}N_2O_4$	380	25	Amide
	Tricoumaroyl spermidine	13.87	584.27	[M+H]+	$C_{34}H_{38}N_3O_6$	$C_{34}H_{37}N_3O_6$	583	105	Amide

Table 3.3 Phytochemicals recovered using LC-MS from the nectar of plants in UK based agri-environment schemes.

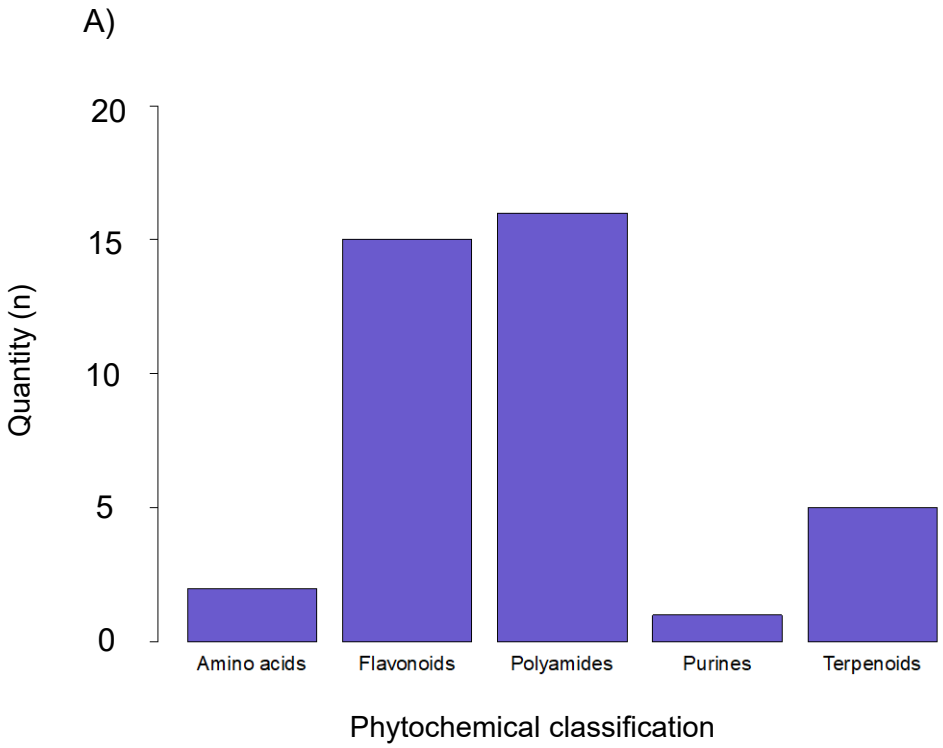
Species	Phytochemical assignment	RT (30 min)	M/Z	Ion (I)	Formula (I)	Formula	MW	PPM	Phytochemical type
Centaurea nigra	3, 8-di-Acetoxyzaluzanin D	12.98	347.14902	[M+H]+	$C_{19}H_{23}O_6$	$C_{19}H_{22}O_6$	346	No UV	Terpenoid
	8-Hydroxyzaluzanin	9.37	280.15466	[M+NH4]+	$C_{15}H_{22}NO_4$	$C_{15}H_{18}O_4$	262	No UV	Terpenoid
	Annuionone B	9.92	223.13	[M+H]+	$C_{13}H_{19}O_3$	$C_{13}H_{18}O_3$	221	11	Terpenoid
	4-7, Megastigmane glycopyranoside	11.57	371.2	[M+H]+	$C_{19}H_{31}O_7$	$C_{19}H_{30}O_7$	370	32	Terpenoid
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	14.81	787.28	[M+H]+	$C_{46}H_{49}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	89	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	15.03	787.3	[M+H]+	$C_{46}H_{49}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	89	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	15.23	787.3	[M+H]+	$C_{46}H_{49}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	89	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	15.45	787.29	[M+H]+	$C_{46}H_{49}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	89	Amide
	N1,N5,N10,N14-Tetra-trans-p-coumaroylspermine	15.67	787.26	[M+H]+	$C_{46}H_{49}N_4O_8$	$C_{46}H_{50}N_4O_8$	786	89	Amide
Lotus corniculatus	Phenylalanine	3.15	166.09	[M+H]+	$C_9H_{12}NO_2$	$C_9H_{11}NO_2$	165	65	Amino acid
	Cinnamic acid	3.15	149.16	[M+H]+	$C_9H_9O_2$	$C_9H_8O_2$	148	65	Carboxylic acid
	Unknown	6.85	400.12	[M+H]+	N/A	N/A	N/A	N/A	Unknown
	Unknown	9.88	501.13	[M+H]+	N/A	N/A	N/A	N/A	Unknown
Malva moschata	Gentianidine	10.65	164.07	[M+H]+	$C_9H_{10}NO_2$	$C_9H_9NO_2$	163	15	Alkaloid
	Unknown	11.62	163.13	[M+H]+	$C_8H_{19}O_3$	$C_8H_{18}O_3$	162	9	Aliphatic alcohol
	Abscisic acid	14.02	247.05	[M+H]+	$C_{15}H_{19}O_3$	$C_{15}H_{18}O_3$	246	125	Terpinoid
Onobrychis viciifolia	Caffeine	6.11	195.09	[M+H]+	$C_8H_{11}N_4O_2$	$C_8H_{10}N_4O_2$	194	39	Alkaloid
	Phenylalanine	3.08	166.09	[M+H]+	$C_9H_{12}NO_2$	$C_9H_{11}NO_2$	165	74	Amino acid

	Unknown	9.14	311.21	[M+H]+	$C_{20}H_{27}N_2O$	$C_{20}H_{26}N_2O$	310	N/A	Alkaloid
Prunella vulgaris	Euperfolin	12.26	365.06	[M+H]+	$C_{20}H_{29}O_6$	$C_{20}H_{28}O_6$	364	12	Dilactone
Ranunculus acris	Sophoraflavonoloside	8.39	611.1608	[M+H]+	$C_{27}H_{31}O_{16}$	$C_{27}H_{30}O_{16}$	610	16	Flavonoid
Rhinanthus minor	Unknown	6.86	371.22	[M+H]+	$C_{19}H_{33}NO_6$	$C_{19}H_{32}NO_6$	370	N/A	Alkaloid
	N-(E)-Feruloyl-3-*O*-methyldopamine	6.44	344.18	[M+H]+	$C_{19}H_{22}NO_5$	$C_{19}H_{21}NO_5$	343	No UV	Flavonoid
	Unknown	13.29	318.08	[M+H]+	$C_{17}H_{20}NO_5$	$C_{17}H_{19}NO_5$	317	N/A	Alkaloid
Trifolium pratense	Unknown	7.51	388.19	[M+H]+	$C_{18}H_{29}O_9$	$C_{18}H_{28}O_9$	387	N/A	Unknown
	Quercetin-3-*O*-arabinoside (Avicularin)	13.33	435.14	[M+H]+	$C_{20}H_{19}O_{11}$	$C_{20}H_{18}O_{11}$	434	43	Flavonoid
Vicia sativa	Dicoumaroyl putrescine	13.2	381.13	[M+H]+	$C_{22}H_{25}N_2O_4$	$C_{22}H_{24}N_2O_4$	380	11	Amide

3.3 Results

3.3.1 Identification of secondary metabolites from AES plants

From the nine angiosperm species included in AES planting strategies that bumblebees are known to forage on, 41 phytochemicals were identified from pollen samples and 27 were identified from nectar samples using LC-MS (Figure 3.1), inclusive of isomers (Table 3.2 & Table 3.3). Of these, four were selected to be trialed in the *C. bombi in vitro* investigation due to their putative biological activity.



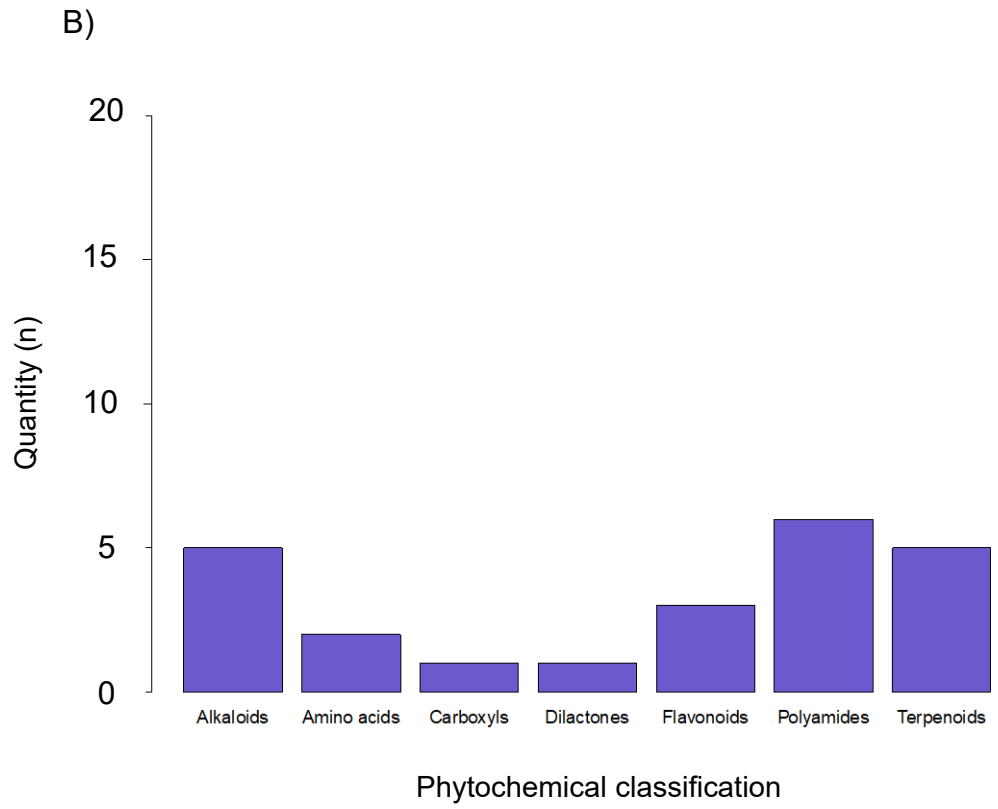


Figure 3.1 The quantity of different classes of phytochemicals recovered from both the pollen (A) and nectar (B) of AES angiosperms that bumblebees are known to forage on.

3.3.2 Testing the biological activity of AES phytochemicals on *Crithidia bombi in vitro*

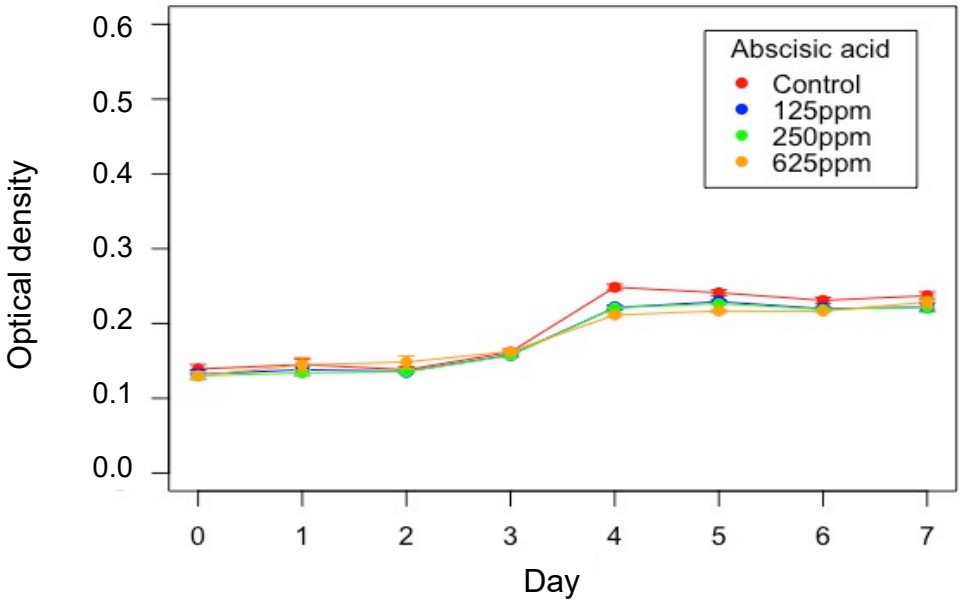
3.3.2.i *Crithidia bombi* optical density data

For the optical density investigation each phytochemical was screened over seven days across two *C. bombi* parasite strains. Across all phytochemicals both strains of *C. bombi* had a stepwise pattern of increased growth between day 4 and 6, before plateauing between day 6 and 8 (Figure 3.2). There was no significant relationship between abscisic acid (LMM, $F_{1,378} = 2.57$, $P = 0.1$), biochanin A (LMM, $F_{1,378} = 3.52$, $P = 0.06$) or TCS (LMM, $F_{1,378} = 2.11$, $P = 0.14$) and *C. bombi* growth (Figure 3.2). However, there was a significant relationship between caffeine (LMM, $F_{1,378} = 3.67$, $P = 0.05$) and *C. bombi* growth. Caffeine increased *C. bombi* growth at lower concentrations, before reducing *C. bombi* growth at higher concentrations.

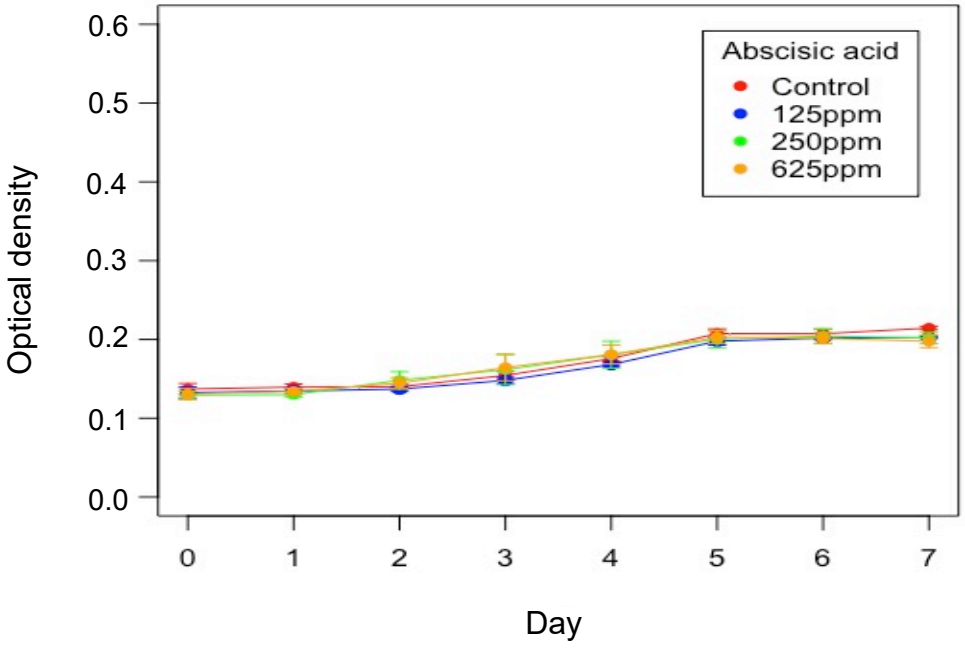
For abscisic acid and TCS the covariate 'day' had no significant effect on optical density data (LMM, abscisic acid $F_{1,378} = 6.64$, $P = 0.01$, TCS $F_{1,378} = 0.06$, P

= 0.8), whereas ‘day’ did have a significant positive effect on the optical density data for both biochanin A and caffeine (LMM, biochanin A $F_{1,378}$ = 4.30, P = 0.05, caffeine $F_{1,378}$ = 46.43, P = 0.01). The random effect ‘parasite strain’ had no significant effect on optical density data for abscisic acid, caffeine or TCS (abscisic acid P = 0.39, caffeine P = 0.6, TCS P = 0.07), however ‘parasite strain’ did have a significant effect on optical density data for biochanin A (P = 0.05), consequently there is a significant difference in optical density readings between the two parasite strains.

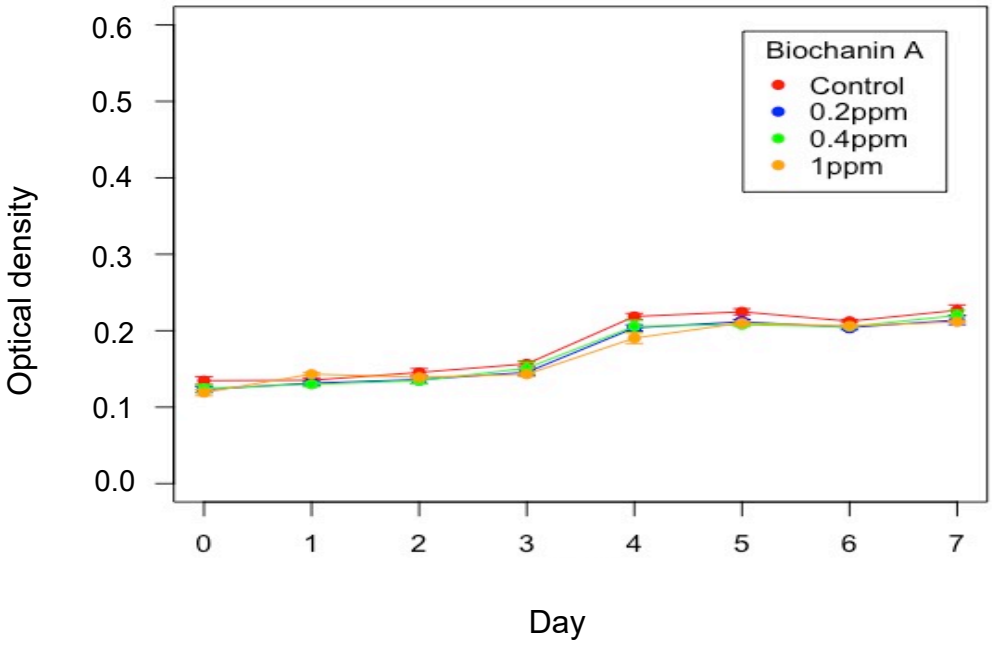
A)



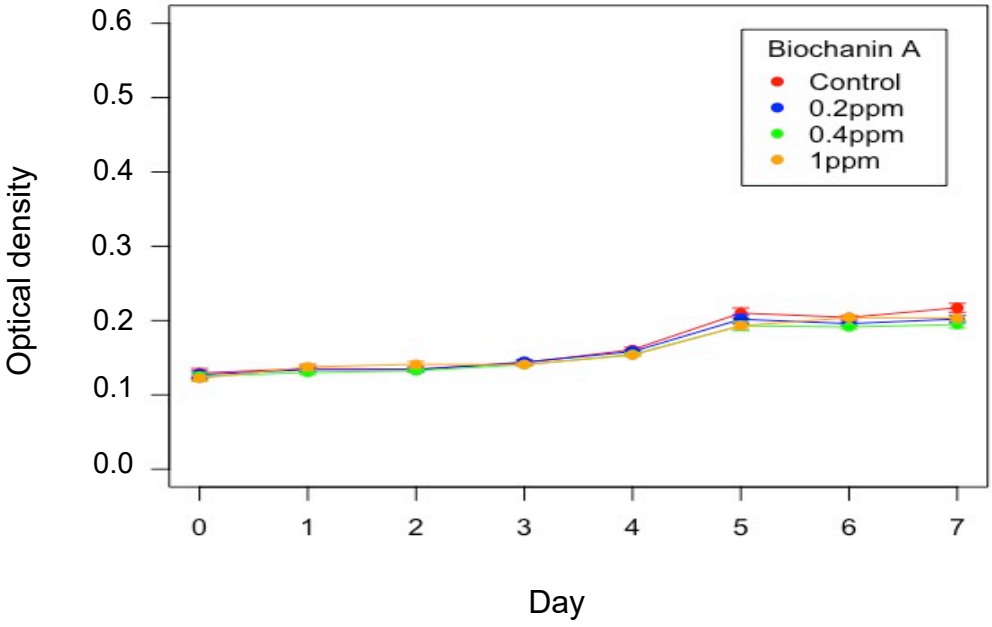
B)



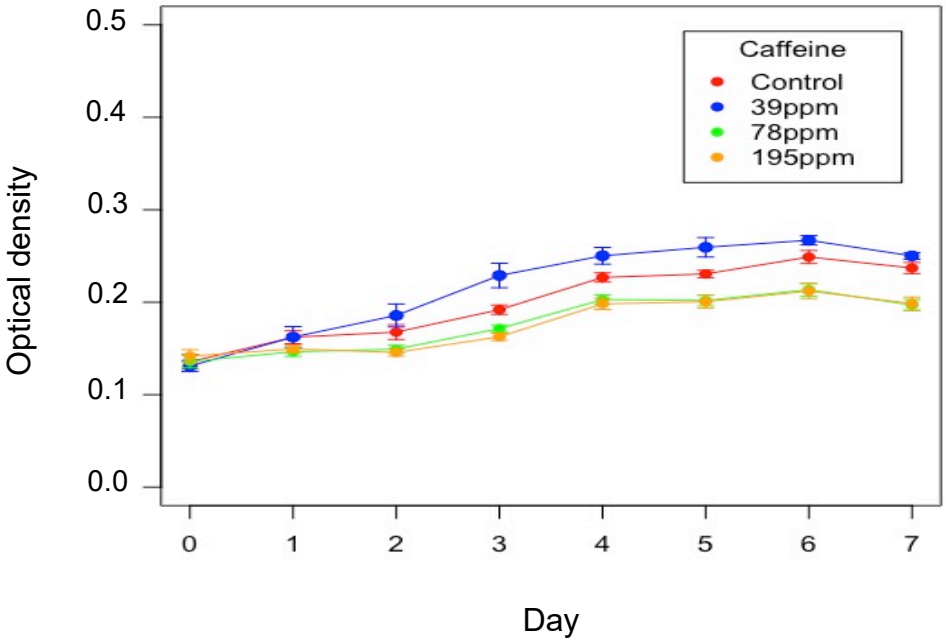
C)


Biochanin A
Control
0.2ppm
0.4ppm
1ppm
Optical density
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
1
2
3
4
5
6
7
Day

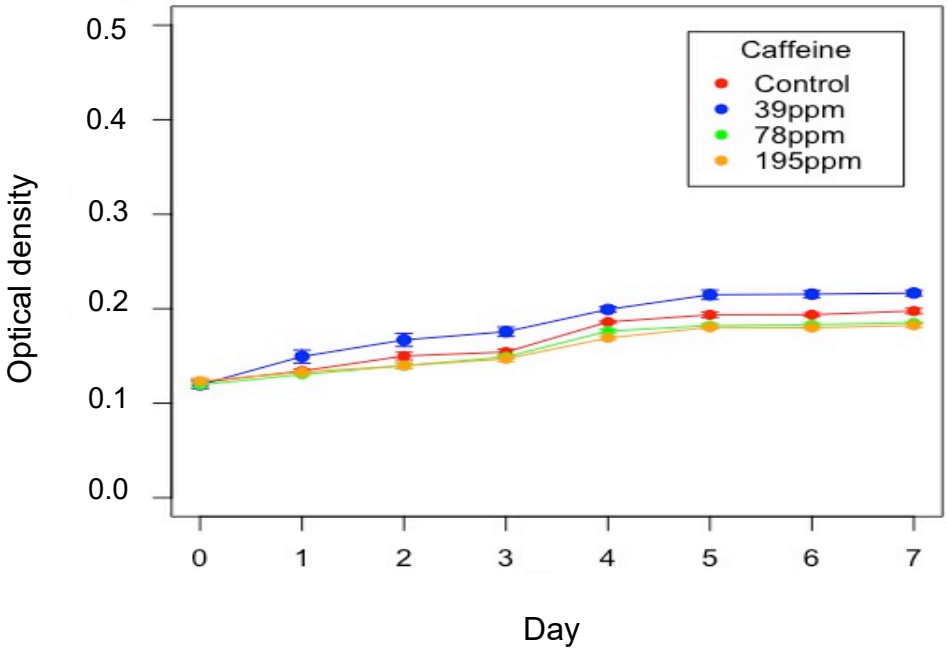
D)


Biochanin A
Control
0.2ppm
0.4ppm
1ppm
Optical density
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
1
2
3
4
5
6
7
Day

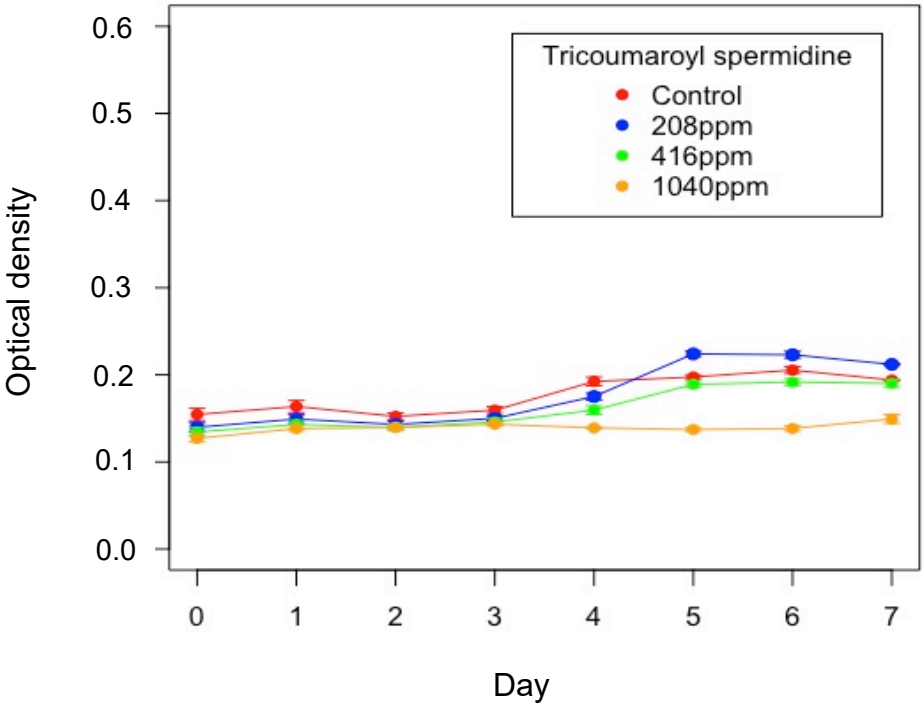
E)


Caffeine
Control
39ppm
78ppm
195ppm
Optical density
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Day

F)


Caffeine
Control
39ppm
78ppm
195ppm
Optical density
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Day

G)


Tricoumaroyl spermidine
Control
208ppm
416ppm
1040ppm
Optical density
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
1
2
3
4
5
6
7
Day

H)

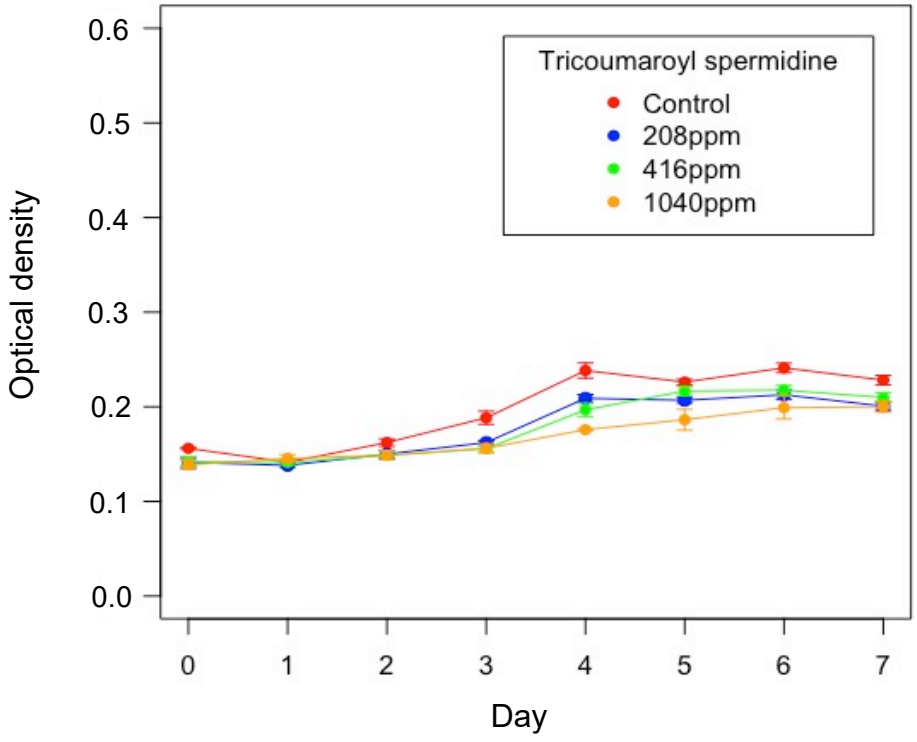
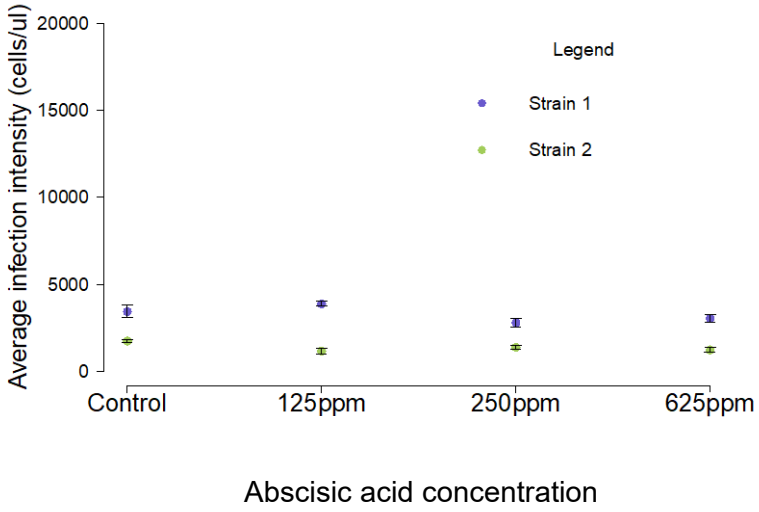

Tricoumaroyl spermidine
Control
208ppm
416ppm
1040ppm
Optical density
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
1
2
3
4
5
6
7
Day

Figure 3.2 Optical density readings showing the effect of each phytochemical on *C. bombi* growth across strain 1 & 2 (mean ± SEM). Abscisic acid (A+B), biochanin A (C+D) and tricoumaroyl spermidine (G+H) had no significant effect on *C. bombi* growth (LMM, abscisic acid, $F_{1,378} = 2.57$, $P = 0.10$, biochanin A, $F_{1,378} = 3.52$, $P = 0.06$ and TCS $F_{1,378} = 2.11$, $P = 0.14$). However caffeine (E+F) did have a significant effect on *C. bombi* growth (LMM, caffeine, $F_{1,378} = 3.67$, $P < 0.05$). Caffeine at lower concentrationsincreased *C.bombi* growth, before reduing *C. bombi* growth at higher concentrations.

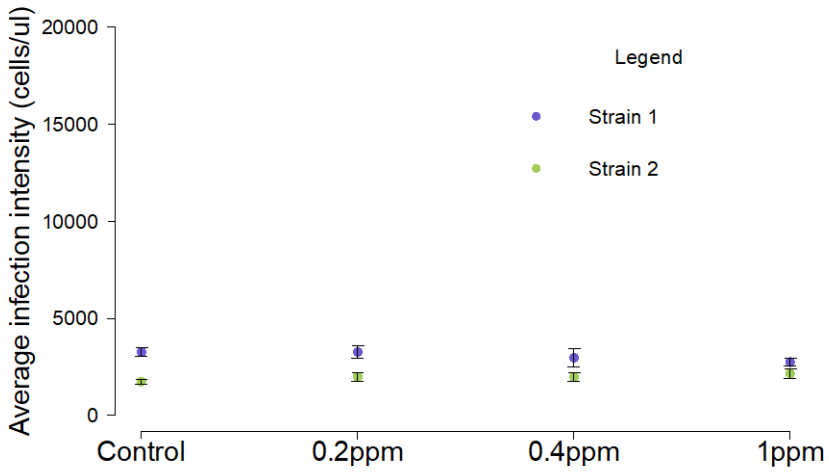
3.3.2.ii *Crithidia bombi* direct parasite count data

A direct parasite count was taken for each replicate using a Neubauer improved haemocytometer at the end of day seven (Figure 3.3). There was no significant relationship between abscisic acid (LMM, $F_{1,44} = 2.00$, $P = 0.16$) and biochanin A (LMM, $F_{1,44} = 3.54$, $P = 0.6$) treatments on *C. bombi* parasite count. In contrast, both caffeine (LMM, $F_{1,44} = 12.54$, $P < 0.001$) and TCS (LMM, $F_{1,44} = 4.72$, $P = 0.03$) had a significant effect on *C. bombi* parasite count data.Interestingly, in the direct parasite count dataset the random effect 'parasite strain' had a significant effect across all replicates (abscisic acid $P = 0.001$, biochanin A $P = 0.04$, caffeine $P = 0.001$, TCS $P = 0.001$), which was not identified in the optical density dataset. Consequently there is a difference in the direct parasite count readings between the two parasite stains, suggesting that they may have different growth dynamics. The phytochemical caffeine, across both strains, at a concentration of 39ppm had a significant positive effect on *C. bombi* parasite count, before having a statistically negative effect at concentrations of 79 and 195ppm (one-way ANOVA, strain one $F_{3,20} = 323.45$, $P < 0.01$ and strain two $F_{3,20} = 84.04$, $P < 0.01$). TCS only had a statistically significant negative effect on parasite strain one at a concentration of 1040ppm (one-way ANOVA, strain one $F_{3,20} = 5.24$, $P = 0.007$).

A)

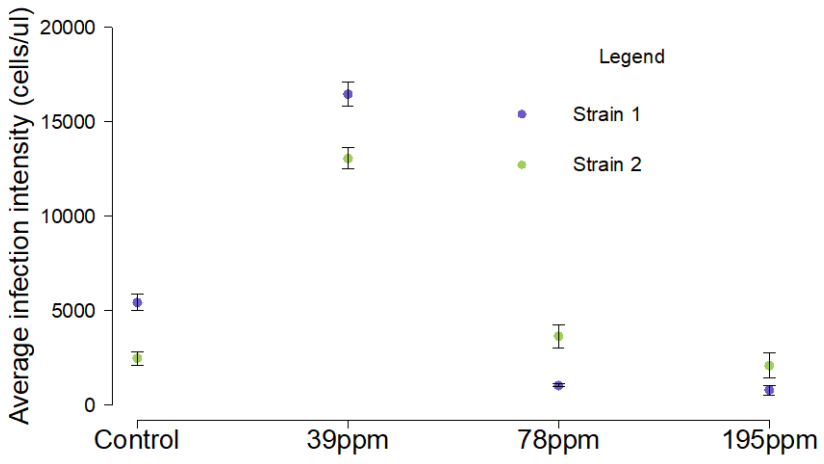


B)



Biochanin A concentration

C)



Caffeine concentration

D)

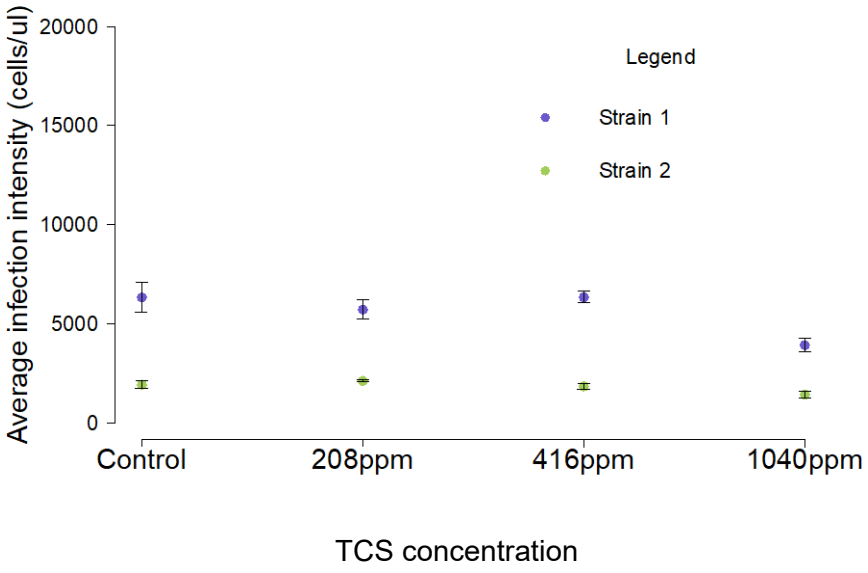


Figure 3.3 Direct parasite count data for the effect of abscisic acid (A), biochanin A (B), caffeine (C) and tricoumaroyl spermidine (TCS) (D) on two strains of *C. bombi* after seven days of continuous exposure (mean ± SEM). Abscisic acid and biochanin A (LMM, abscisic acid, $F_{1,44} = 2.00$, $P = 0.16$ and biochanin A, $F_{1,44} = 3.54$, $P = 0.06$) had no significant effect, whereas caffeine and TCS (LMM, caffeine, $F_{1,44} = 12.54$, $P = 0.001$ and TCS, $F_{1,44} = 4.72$, $P < 0.001$) had a significant effect on *C. bombi* parasite count data. Similarly to the optical density dataset caffeine increased *C. bombi* growth at lower concentrations before reducing *C. bombi* growth at higher concentrations. Whereas TCS only reduced *C. bombi* growth at its highest concentration.

3.4 Discussion

My results demonstrate that plants currently used in UK based agri-environment schemes (AES) contain bioactive phytochemicals in both their pollen and nectar. My results also show that two of these phytochemicals have a direct effect on the *in vitro* growth rate of *C. bombi*, an important internal parasite of bumblebees. Therefore schemes such as AES, which can increase the diversity of phytochemicals that pollinators encounter, may also indirectly impact on bumblebee health.

Pollen and nectar, the primary food source for bumblebees, contain secondary metabolites (Adler 2000, Stevenson *et al*. 2017) and these may have negative impacts on bumblebees (Cook *et al*. 2013). Here, using high performance LC-MS I was able to identify 62 phytochemicals in both the pollen and nectar from plants that bumblebees are known to forage on from UK-based AES. Given that bumblebees undertake numerous daily foraging bouts (Sladen 1912), it is likely that both foragers and the developing colony in areas where these AES schemes are applied are continuously exposed to these phytochemicals, and therefore understanding their impact on bumblebee health is fundamental to the assessment of the effectiveness of AES. The primary goal of AES is to enhance agricultural landscapes and to support biodiversity (EU Common Agricultural Policy 2015, Natural England 2017). Within Europe, AES have been shown to have positive effects on biodiversity (Kleijn *et al*. 2007). More specifically AES have led to an increase in ecosystem services, such as pollination (Whittingham 2011), and have increased arthropod numbers (Kleijn & Sutherland 2003). However, there are subtle differences in how these schemes are implemented across the EU (Kleijn & Sutherland 2003) and consequently, due to the high budgets set aside for AES (Science for Environment Policy 2017) the effectiveness of these schemes is under constant assessment. It has been shown that although AES prescriptions add resources to arable landscapes, their national UK nectar contribution is low (Baude *et al*. 2016). However, plants that are included in AES prescriptions, which are targeted at improving pollinator diversity, have been selected to include a diverse mix of wildflowers (Pywell *et al*. 2011(b)) that include late forage species (Pywell *et al*. 2011(a)). These wildflower mixes that are high in pollen and nectar producing flowers have been shown to increase bumblebee species richness (Pywell *et al*. 2006, Carvell *et al*. 2007) and experimental work has shown that florally enriched landscapes have a direct fitness benefit for bumblebees (Carvell

et al. 2017). Guidance on the current selection of wildflowers included in AES seed mixes has come from scientific research (Pywell *et al*. 2011(a), Dicks *et al*. 2015) and consequently these schemes can be viewed as dynamic. As phytochemicals are ubiquitous (Adler 2000, Palmer-Young *et al*. 2018) and can impact pollinators (Tiedeken *et al*. 2016) any schemes that increase phytochemical diversity and abundance must be effectively assessed to address the potential impact on target pollinators.

Previous work has identified that phytochemicals in floral rewards are bioactive and can have negative impacts on bees (Tiedeken *et al*. 2016, Arnold *et al*. 2014), although others have been shown to have positive fitness benefits by reducing parasite loads in bumblebees (Manson *et al*. 2010, Richardson *et al*. 2015). Whilst these data appear to be in conflict, they actually highlight the diverse nature of bioactive phytochemicals, and are consistent with my findings. The phytochemicals abscisic acid and biochanin A had no measureable effect on *C. bombi* growth, while caffeine and TCS both had an impact on *C. bombi* growth. It is noteworthy that not all phytochemicals have detectable bioactivity and previous work has shown that *C. bombi* is able to develop resistance to bioactive phytochemicals (Palmer-Young *et al*. 2017). Therefore it is likely that some phytochemicals ingested by bumblebees during foraging bouts may have no measurable effect against trypanosome parasites, as I have shown here. In contrast, caffeine is a methylxanthine alkaloid, which has been identified in legume species (Islam *et al*. 2012) and has been recovered from nectar (Kretschmar & Baumann 1999). This phytochemical is well known for its bioactive properties (Raj & Dhala 1965) and here has been identified using high performance LC-MS in the nectar of *O. viciifolia* at a concentration of 39ppm. When investigating the *in vitro* effect of caffeine at its recovered natural concentration, the growth rate, measured using optical density, of *C. bombi* was significantly increased versus the control treatment. This increase in growth rate was identified over both parasite strains, indicating that this relationship occurs across genotypes. Direct parasite count data collected at the end of the experiment supported this conclusion. In contrast, at higher concentrations (78ppm and 195ppm) caffeine had an inhibitory effect on *C. bombi* growth. Interestingly, this relationship where caffeine increases growth at lower concentrations before inhibiting growth at higher concentrations has previously been identified in other microorganisms (Gokulakrishyan & Gummadi 2006, Gutiérrez *et al*. 2013). This may be a function of *in vitro* caffeine increasing the

metabolic rate of *C. bombi*, before becoming toxic at higher concentrations. It is worth noting that a caffeine concentration of 98 ppm is ecologically relevant for *Citrus spp*. (Kretschmar &Baumann 1999) and this is in excess of my two times ecologically relevant caffeine concentration. Consequently, this highlights that a single phytochemical may have multiple impacts on bumblebee health depending on the concentration a forager encounters in the wild, but how continuous ingestion relates to the sequestration of a phytochemical within a bumblebee remains unclear. The amide TCS, recovered from the pollen of *L. corniculatus*, has previously been shown to have antimicrobial activity (Walters *et al*. 2001). However, here TCS only had a significant effect on *C. bombi* growth in parasite strain two at five times its ecologically relevant concentration; again, this was supported with the direct parasite count data. TCS is a common constituent of pollen and it is possible that *C. bombi* has evolved to deal with ecologically relevant concentrations of this compound.

I would note that this work only investigates the impact of a single phytochemical on *C. bombi* growth. Previous work has shown that combinations of phytochemicals tested *in vitro* on *C. bombi* may have synergistic effects (Palmer-Young *et al*. 2017). Therefore, it is likely that bumblebees foraging in AES enhanced land will encounter a diverse suite of phytochemicals at a range of concentrations and how these phytochemicals interact and impact on bumblebee health requires further investigation. In addition I would note that this work tests the impact of phytochemicals on *C. bombi* growth *in vitro*. It is likely that results may differ in an *in vivo* investigation due primarily to the hosts immune response.

To my knowledge this investigation provides the first results on the *in vitro* impact of phytochemicals on *C. bombi* growth that includes both optical density and parasite count data. The optical density dataset provides an insight into the growth dynamics of *C. bombi* over the experimental timeframe. Primarily it identifies that *C. bombi* has a measureable stepwise growth increase between day 4 and 6, which is consistent with previous *in vivo* findings (Logan *et al*. 2005). In contrast, the parasite count data provides a clear insight into the replication of *C. bombi* during the experimental timeframe. Previous work (Palmer-Young *et al*. 2016, Palmer-Young & Thursfield 2017, Palmer-Young *et al*. 2017) has used optical density as a proxy for parasite growth, which may limit the interpretation of results. Here, the optical density dataset was unable to differentiate any effect of the two separate parasite strains on *C. bombi* growth, however, the parasite count dataset clearly identified the effect that

different strains have on the growth of *C. bombi* when challenged with phytochemical stressors. Moreover, marginal differences in optical density data can lead to large differences in parasite count data, as seen in my caffeine dataset. Elucidating how optical density relates to final parasite counts provides a more holistic view of how phytochemical stressors impact *C. bombi* and how this may then impact on infection success in bumblebees (Ruiz-González & Brown 2006).

Of the 62 phytochemicals identified here from AES, only four were selected for use in the *in vitro* investigation. Primarily phytochemicals were selected for their known biological activity, but also for their availability. This highlights a limitation in both the knowledge of the bioactive properties of common phytochemicals and also the ability to access compounds that are not available commercially. It is likely that other untested phytochemicals identified within this investigation may also have an impact on bumblebee or pollinator health.

The main priority of AES is to enhance agricultural land and improve biodiversity (EU Common Agricultural Policy 2015, Natural England 2017). Prescriptions set out by AES to improve insect pollinator abundance and diversity have been shown to improve pollination services (Whittingham 2011), but also to increase pollinating insect diversity and abundance (Grass *et al*. 2016). More specifically, florally enriched landscapes have a direct fitness benefit for bumblebees (Carvell *et al*. 2017). However, my results show that plants contained in AES planting strategies contain bioactive phytochemicals and that these may have an indirect impact on bumblebee health by increasing or reducing parasite loads. Consequently, my results highlight that determining the chemical profile of forage plants is key to understanding how landscapes and schemes such as AES will impact on pollinator health.

Chapter 4

The effect of a floral isoflavone on the bumblebee parasite *Nosema bombi*

Abstract

Emerging infectious diseases pose a major threat to a diverse range of global fauna. More specifically, emerging fungal diseases have been linked to population declines in both mammals and amphibians. Bumblebees, a key group of pollinators, are also threatened with emerging fungal diseases, such as infection with the microsporidian parasite *Nosema bombi*. This parasite has severe fitness consequences for its bumblebee host and has been linked to dramatic declines in a number of North American bumblebee species. Consequently, elucidating the interaction between *N. bombi* and its bumblebee hosts is of critical importance. Here I provide the first detailed methodology for individual, quantifiable *N. bombi* inoculations in developing *Bombus terrestris* larvae, which resulted in a 42-51% infection success rate, post-eclosure in control bumblebees. In addition, as phytochemicals have been shown to ameliorate the impact of trypanosome disease in bumblebees, I tested the putative antifungal activity of the floral isoflavone biochanin A on *N. bombi* in three novel *in vivo* bioassays. Biochanin A reduced *N. bombi* infection intensity and had a significant prophylactic effect in developing *B. terrestris* larvae, and a significant therapeutic effect in adult workers. These results show, for the first time, that phytochemicals can impact microsporidia infections in bumblebees across different life stages. Consequently investigating ecologically relevant phytochemicals may help mitigate the impact of virulent diseases in an economically important pollinator.

Key words: *Bombus terrestris*, pollinator health, microsporidia, biochanin A, phytochemical, secondary metabolite, red clover, *Trifolium pratense*

4.1 Introduction

Emerging infectious diseases (EIDs) are classed as pathogens that increase their geographic range, incidence or virulence (Jones *et al*. 2008) and which may pose a substantial threat to global biodiversity (Daszak *et al*. 2000). More specifically, emerging fungal diseases have been linked to population collapses in a range of fauna, most notably the global spread of chytrid in amphibians and white-nosed syndrome in bats (Berger *et al*. 1998 & Leopardi *et al*. 2011, respectively). Typically, emerging fungal diseases enter a novel host or environment where susceptible individuals have not evolved to cope with a particular fungal stressor (Fisher *et al*. 2011). Consequently, naïve individuals are unable to combat a novel fungal infection effectively, which may lead to increased virulence in a given host-parasite system (Fisher *et al*. 2013). The spread of such diseases is not limited to amphibians and mammals, with pollinating insects such as bumblebees also under threat from EIDs (Fürst *et al*. 2014), including emerging fungal diseases (Cameron *et al*. 2016, Brown 2017). Bumblebees are relatively simple eusocial insects (Sladen 1912, Wilson 1971) that provide essential, economically important pollination services (Breeze *et al*. 2011). However, a number of bumblebee species are undergoing drastic, global population declines (Williams 1982, Fitzpatrick *et al*. 2007, Williams & Osborne 2009, IUCN 2015) and these may be driven, in part, by an increase in parasite prevalence (Cameron *et al*. 2011, Cameron *et al*. 2016).

The high population densities and low genetic variability of social insects are ideal for parasite transmission (Schmid-Hempel 1998, Barribeau *et al*. 2015, but see also Van Baalen & Beekman 2006) and bumblebees encounter a range of detrimental parasites during their annual colony cycle (Schmid-Hempel 1998). One such parasite that has a negative impact on the fitness of its bumblebee host is *Nosema bombi* (Fantham & Porter 1914, Otti & Schmid-Hempel 2007, Rutrecht & Brown 2008). This microsporidian parasite infects individuals *per os* and the infection manifests itself in the gut, malpighian tubules and fat bodies of its host (Fantham & Porter 1914). Whilst the environmental prevalence of *N. bombi* is estimated to be lower than that of other well-studied bumblebee parasites (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014) it is potentially more deleterious to wild bumblebee populations. Previous work has linked the population declines and range collapses of a number of North American bumblebee species to *N. bombi* infection (Cameron *et*

al. 2011, Cameron *et al*. 2016). In addition there is correlative evidence to show that individuals infected with *N. bombi* forage less efficiently (Shykoff & Schmid-Hempel 1991) and infected *B. terrestris* field colonies were shown not to produce any reproductives (Otti & Schmid-Hempel 2008). Interestingly, work with the prevalent bumblebee parasite *Crithidia bombi* (Gorbunov 1987) has highlighted that phytochemicals in nectar may reduce parasite intensity (Manson *et al*. 2010, Richardson *et al*. 2015) and may therefore indirectly improve bumblebee health. This poses the question of whether phytochemicals found in floral rewards may have a similar effect on microsporidian infections in bumblebees.

Plant secondary metabolites are synthesized in plant tissues and are classified as any compounds that are not used for primary development. These metabolites are translocated, albeit in lower concentrations, into floral rewards (Baker & Baker 1975, Adler 2000, Stevenson *et al*. 2017). Whilst these phytochemicals may be beneficial for plant reproductive fitness (Adler 2000) they may also have a negative impact on foraging bees (Cook *et al*. 2013, Arnold *et al*. 2014, Tiedeken *et al*. 2016) and thus the presence of these phytochemicals represents a fitness trade off for entomophilious plants (Gegear *et al*. 2007). However, some phytochemicals have been shown to have antimicrobial properties (Cowan 1999) and may therefore have an indirect positive impact on bumblebee health by reducing parasite loads (Manson *et al*. 2010, Barrachi *et al*. 2015, Richardson *et al*. 2015). Strategies that increase floral abundance and diversity within homogenous landscapes, such as AES (EU Common Agricultural Policy 2015), may also indirectly increase the abundance of bioactive phytochemicals that pollinators encounter. Consequently, land enhanced by such strategies makes an excellent candidate to sample for ecologically relevant, bioactive phytochemicals that may impact bumblebee health.

Red clover (*Trifolium pratense*) is a legume species that bumblebees are known to forage on and is included in AES wildflower prescriptions (Pywell *et al*. 2011, Natural England 2017). Biochanin A, an isoflavone, has been isolated from the reproductive floral tissues and pollen of *T. pratense* using liquid chromatography and mass spectrometry (LC-MS)(Wu *et al*. 2003, Saviranta *et al*. 2008, this thesis 3.3.1). Interestingly, biochanin A has a planar structure due to the positioning of its hydroxyl groups and compounds with this conformation have been shown to compete for fungal cell wall receptor sites (Weidenbörner *et al*. 1990). Consequently, biochanin A

may have antifungal properties (Rojas *et al*. 2006), which makes it an ideal phytochemical to test for biological activity on *N. bombi*.

To my knowledge no previous work has investigated the effect of bioactive phytochemicals on *N. bombi* infection in bumblebees. Given that *N. bombi* is infective to developing bumblebee larvae and that the infection persists though pupation (Schmid-Hempel 1998, Rutrecht & Brown 2007) I have designed three novel *in vivo* bioassays to elucidate the antifungal activity of biochanin A. During the larval stages of bumblebee development I address the following questions 1) Is *N. bombi* infection intensity reduced in eclosed workers that were fed biochanin A in sugar water before inoculation? and 2) Is *N. bombi* infection intensity reduced in eclosed workers that were fed biochanin A in sugar water after inoculation as larvae?, By altering the timing of inoculations in this way, I was able to identify if biochanin A had any prophylactic or therapeutic properties on *N. bombi in vivo*. Finally I investigated whether 3) *N. bombi* infection intensity is reduced in adult workers that are fed biochanin A in sugar water.

4.2 Methodology

4.2.1 Chemical analysis of red clover

For methods describing the chemical analysis of the pollen and nectar of AES plants please refer to section 3.2.1 of this thesis. The isoflavone biochanin A was identified in the *T. pratense* pollen sample using *m/z* 284 and rt = 17.9 min. In addition, I was able to quantify a concentration in parts per million (ppm) of biochanin A in the pollen sample, using an appropriate biochanin A standard, as 0.1ppm. As this investigation is a novel proof of principle work I opted to increase the biochanin A concentration for my experimental trials to 20ppm to ensure any effect would be detected in my bioassays. This concentration is within the natural variation recorded from *T. pratense* floral tissue (Saviranta *et al*. 2008).

4.2.2 Colony provenance

Eight *Bombus terrestris audax* colonies (hereafter referred to as donor colonies), containing a queen, brood and a mean of 45 (± 6.5 S.E.) workers, were obtained from

Biobest, Belgium. Colonies were kept in a dark room at 26°C and 50% humidity (red light was used for any colony manipulation). To ensure colonies were healthy and developing normally they were monitored for 7 days prior to use in any experimental procedures. This included randomly screening 10% of the workers every two days, from each colony, for parasitic infections in faeces using a phase-contrast microscope set to ×400 magnification. No infections were identified in any of the eight donor colonies.

Micro-colonies were established by removing 5 patches of brood containing approximately 15 developing larvae (growth stage L2-3), from each of the eight donor colonies. Each of these patches of brood was placed in an individual 14×8×5.5cm acrylic box. The micro-colonies were each provisioned with *ad libitum* pollen and sugar water, and 3 workers from their original donor colony to provide brood care. All pollen used throughout the experiment was irradiated to remove any microbes. Prior to being entered into the experiment all brood-caring workers were individually marked using a coloured, numbered Opalith tag and recorded.

4.2.3 Artificial inoculation of *B. terrestris* larvae with *N. bombi*

To elucidate the effect of biochanin A on *N. bombi* required all experimental larvae to be inoculated. A wild *B. terrestris* queen that was naturally infected with *N. bombi* was caught from Windsor Great Park, UK (SU992703) in 2016. The infected queen's gut was isolated by dissection and homogenized in 0.01M NH_4Cl. The resulting spore solution was centrifuged at 4°C for 10 minutes at 5000 rpm to isolate and purify the spore pellet as described in Rutrecht & Brown (2008). The pellet was resuspended in 0.01M NH_4Cl and the *N. bombi* concentration was calculated using a Neubauer improved haemocytometer. To confirm the presence of *N. bombi,* and to ensure the microsporidia was not *N. ceranae,* a sample of the inoculum was subjected to PCR using primers and the protocol outlined in Erler *et al.* (2012) (Appendix 9.2). The inoculum was then stored at -80°C until required.

A larval *N. bombi* inoculant was prepared by combining inverted sugar water and pollen (3:1) to create an artificial worker feed as outlined in Folly *et al*. (2017). This was then combined in equal proportions (100µl:100µl) with the *N. bombi* inoculum to create an experimental inoculant. Prior to any larval inoculation, workers

from each micro-colony were removed for an hour. This resulted in larvae having no access to food and therefore experimental inoculation would be more likely to elicit a feeding response. Larvae were then assigned to one of two feeding trials.

4.2.4 Does biochanin A impact *N. bombi* infection in developing *B. terrestris* larvae?

To test the effect of biochanin A on developing larvae, 32 micro-colonies, as described above, were used. In the first feeding trial (n=16 micro-colonies, 2 per donor colony) prior to inoculation, control larvae were kept in their original micro-colonies (n=8) and provided *ad libitum* pollen and sugar water. In the experimental groups (n=8) *ad libitum* pollen and sugar water containing biochanin A at 20 ppm was provided for 7 days. Biochanin A was added to sugar water using 4ml of 40% MeOH as a solvent per litre, and control colonies also had 4ml of 40% MeOH added per litre of sugar water. After seven days both experimental and control larvae were artificially inoculated with 50,000 *N. bombi* spores in 4.3µl of inoculant using a 20µl pipette, as described above. The spore concentration of the inoculum is within ecological relevant values for *N. bombi* spores in faeces and has been shown to be a concentration that is infective to developing *Bombus terrestris* brood (Rutrecht & Brown 2008, AF unpublished pilot work). The larvae were left to consume the inoculum for 30 minutes, before being returned to their micro-colony. This bioassay enabled the detection of any prophylactic effects that biochanin A may have against *N. bombi* infection in developing larvae.

In a simultaneous feeding trial, larvae were each inoculated with 50,000 spores in 4.3µl of experimental inoculant, as described above, using a 20µl pipette prior to being entered into a feeding regime. As before larvae were left for 30 minutes to consume the inoculum. The inoculated larvae were returned to their respective micro-colonies with brood-caring workers. Each control micro-colony (n=8) was provisioned with *ad libitum* pollen and sugar water. However, in the experimental groups (n=8) *ad libitum* pollen and sugar water containing biochanin A at 20 ppm was provided for 7 days. Biochanin A was added to sugar water using 4ml of 40% MeOH as a solvent per litre, control colonies also had 4ml of 40% MeOH added per litre of sugar water. This bioassay was designed to test the therapeutic effect of biochanin A on *N. bombi* during larval development.

In both feeding trials larvae were allowed to develop naturally and pupate in their respective micro-colonies. Once eclosed, new workers were marked using a coloured Opalith tag and individually quarantined for 3 days in an inverted plastic cup, which was modified with a hole that enabled a 15ml falcon tube to be inserted. The falcon tube contained control inverted sugar water diluted with double distilled H_2O (50% w/w) that the workers could feed on. A quarantine period was used to ensure that faeces samples were not heavily contaminated with pollen, as this can complicate parasite quantification. At the end of the quarantine period each worker was isolated in a 25ml plastic vial where it provided a faecal sample, which was then collected in a 10 µl glass capillary enabling the volume (µl) to be recorded. Following this each worker's faecal sample was screened for *N. bombi* by microscopic examination using a phase-shift microscope at ×400 magnification. If an infection was identified a Neubauer improved haemocytometer was used to quantify the parasite load. In addition each worker had its thorax measured (mm) three times and averaged, as a proxy for bumblebee size, using a set of Mitutoyo^{tm} digital calipers. Workers were then sacrificed and stored in a labeled Eppendorf tube at -80°C.

4.2.5 Does biochanin A impact *N. bombi* infection intensity in infected *B. terrestris* workers?

As *N. bombi* infections persist through pupation into adulthood, therapeutic foraging could indirectly improve the health of infected workers. Here, eight micro-colonies were established as described above, one for each donor colony. Brood-caring workers were removed and larvae in each micro-colony were inoculated with 50,000 spores in 4.3µl of inoculant using a 20µl pipette. Larvae were left, as before, to consume the inoculant before brood-caring workers were returned. The micro-colonies were provided with *ad libitum* pollen and sugar water and allowed to develop normally. Once they had eclosed, new workers were individually marked and quarantined as before. All eclosed and quarantined workers were screened for *N. bombi* infection by microscopic examination of faeces using a phase-shift microscope at ×400 magnification. All workers that were infected had their initial parasite load taken using a Neubauer improved haemocytometer and were placed into the feeding trial.

Each infected worker was placed into an inverted plastic cup, as described above, which was blindly allocated to one of two feeding regimes. Experimental bees were provisioned with 15ml of sugar water containing biochanin A at 20 ppm and control bees were given 15ml of control sugar water. As before, biochanin A was added to sugar water using 4ml of 40% MeOH as a solvent per litre, control colonies also had 4ml of 40% MeOH added per litre of sugar water. Infected workers were kept under quarantine as described for 7 days. Every two days each worker was removed and a sample of faeces was taken using a 10µl glass capillary tube. This sample was then measured for volume (µl) and screened for *N. bombi* parasite load using a Neubauer improved haemocytometer. After seven days of experimental feeding thorax measures (mm) for each worker were taken three times and averaged, as a proxy for bumblebee size, using a set of Mitutoyo^{tm} digital calipers and were sacrificed and stored in a labeled Eppendorf tube at -80°C. No pollen was provided during the experimental period.

4.2.6 Statistical analysis

All statistical analyses and graphical outputs were undertaken in R open source programming language (R Core Team 2018, Wickham 2009). To analyse the therapeutic and prophylactic effect of biochanin A on *N. bombi* infection intensity (cells/µl) in newly eclosed workers, two separate linear mixed-effects models (LMM) were constructed. Models were constructed in the R package 'lme4' (Bates *et al*. 2015). Both models were constructed using the following parameters. Infection intensity was used as a response variable, with treatment group, thorax width (mm) and faeces volume (µl) as designated covariates. Both models also incorporated donor colony as a random effect. To analyse the effect of biochanin A feeding on adult workers a third LMM model was constructed. Here, infection intensity was selected as a response variable with treatment group, day, thorax width (mm), and faeces volume (µl) as covariates. As before, donor colony was included as a random effect. Models were validated in R by visually checking the normality of residuals, and for overdispersion and collinearity of variables.

4.3 Results

4.3.1 Do phytochemicals impact *N. bombi* prevalence?

In the prophylactic experiment 188 workers successfully eclosed of which 74 had *N. bombi* infections (control n = 48, experimental n = 26) leading to an overall infection success of 39%. In contrast in the therapeutic feeding trial 179 adult workers successfully eclosed, of which 86 had *N. bombi* infections (control n = 56, experimental n = 30) resulting in an overall infection success of 48%. There were no significant differences in infection success proportion between the treatments in either bioassay (prophylactic, $\chi^2 = 0.017$, $P = 0.89$, therapeutic $\chi^2 = 0.138$, $P = 0.71$) (Figure 4.1).

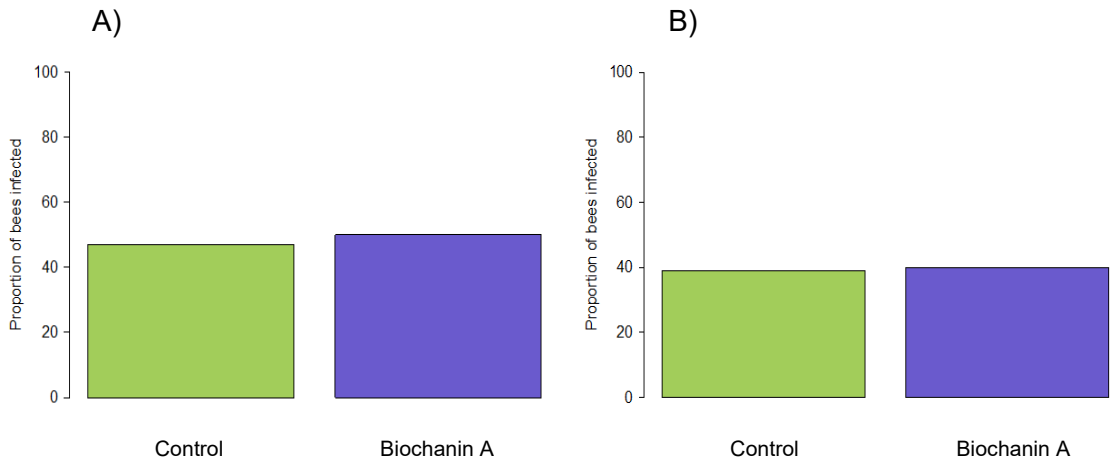


Figure 4.1 *Nosema bombi* infection success in adult *B. terrestris* workers in both the prophylactic (A) and therapeutic (B) bioassays. There was no significant difference between infection success in either treatment (prophylactic, $\chi^2 = 0.017$, $P = 0.89$, therapeutic $\chi^2 = 0.138$, $P = 0.71$).

4.3.2 Does biochanin A impact *N. bombi* infection intensity in developing larvae?

In the prophylactic bioassay, biochanin A treatment had a significant negative effect on *N. bombi* infection intensity (LMM, $F_{1,43} = 6.05$, $P = 0.018$). The covariates thorax width (LMM, $F_{1,43} = 0.04$, $P = 0.824$), faeces volume (LMM, $F_{1,43} = 1.966$, $P = 0.167$), and the random effect colony (LMM, $P = 0.06$) were found to have no significant effect on *N. bombi* infection intensity. In contrast in the therapeutic bioassay, biochanin A treatment did not to have a significant effect on *N. bombi* infection intensity in newly eclosed workers (LMM, $F_{1,51} = 2.2867$, $P = 0.1366$). Similarly, the covariates thorax width (LMM, $F_{1,51} = 0.049$, $P = 0.82$), faeces volume (LMM, $F_{1,51} = 1.81$, $P = 0.18$), and the random effect colony (LMM, $P = 0.7$) had no significant effect on *N. bombi* infection intensity. (Figure 4.2).

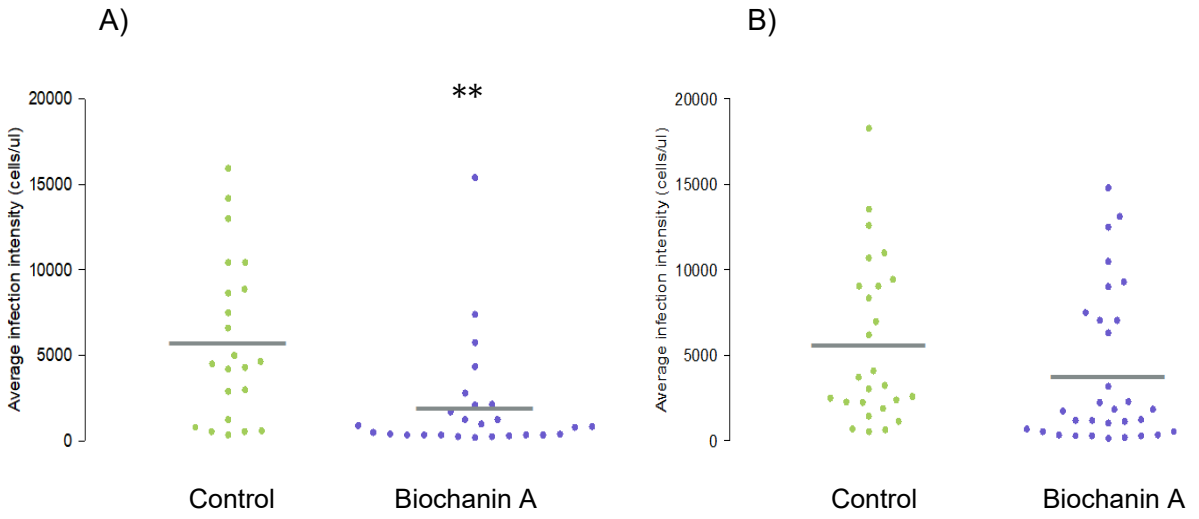


Figure 4.2 Beeswarm plot, used to show the complete spread of data, of *N. bombi* infection intensities in adult *B. terrestris* workers for both the prophylactic ((A), n = 74) and therapeutic ((B), n = 86) bioassays. The sample mean has been marked with a grey bar and statistical differences have been marked with a double asterisk. Biochanin A had a significant prophylactic effect on *N. bombi* infection intensity (LMM, $F_{1,43} = 6.05$, $P = 0.018$).

4.3.3 Does biochanin A impact *N. bombi* infection intensity in *B. terrestris* workers?

In the adult therapeutic investigation 80 workers successfully eclosed, of which 34 were infected with *N. bombi*, giving an infection success rate of 42.5%. However, only 23 workers survived the full duration of the experiment. Both treatment group (LMM, $F_{1,78} = 12.51$, $P < 0.001$) and day (LMM, $F_{1,78} = 71.30$, $P < 0.001$) had significant effects on *N. bombi* infection intensity, with infection intensity increasing over time, but at a significantly lower level in treated individuals. In addition, the random effect colony (LMM, $P = 0.003$) also had a significant effect on *N. bombi* infection intensity. The covariates thorax width (LMM, $F_{1,78} = 1.69$, $P = 0.196$) and faeces volume (LMM, $F_{1,78} = 3.39$, $P = 0.069$) had no significant effect on *N. bombi* infection intensity (Figure 4.3).

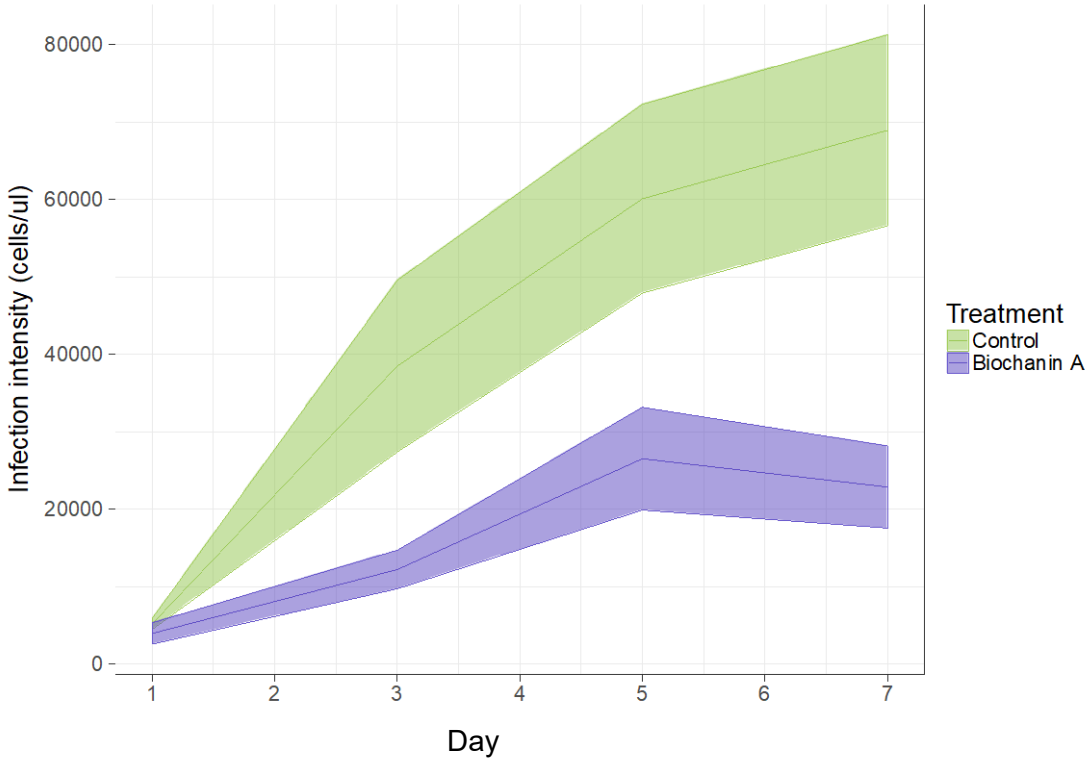


Figure 4.3 The infection intensity (shaded areas represent mean ±SEM) of *N. bombi* in adult *B. terrestris* workers (n = 23) over a seven day period when given a control or biochanin A sugar water supplement. The covariates, treatment (LMM, $F_{1,78} = 12.51$, $P < 0.001$), day (LMM, $F_{1,78} = 71.30$, $P < 0.001$) and the random effect colony (LMM, $P = 0.003$) had a significant effect on *N. bombi* infection intensity.

4.4 Discussion

My results demonstrate that my novel methodology for individual, quantifiable *N. bombi* inoculations resulted in a 42 – 51 % infection success rate in control *B. terrestris* workers. My results also show that consumption of the floral isoflavone biochanin A at 20ppm during larval development has a prophylactic effect, which significantly reduced *N. bombi* infection intensity in newly eclosed workers. Finally my results show that adult *B. terrestris* workers who were infected with *N. bombi* had significantly lower infection intensities when they consumed biochanin A at 20ppm over a period of seven days.

Bumblebees are impacted by a number of detrimental, microbial pathogens (Schmid-Hempel 1998). The most studied of these is *C. bombi* (Gorbunov 1987, Schmid-Hempel & Schmid-Hempel 1993). This trypanosome parasite has high infection success (Otterstatter & Thomson 2007) and has condition dependent virulence (Brown *et al*. 2000). Both of these traits make it an excellent model organism to use in host-parasite investigations. However, the microsporidian *N. bombi* is potentially more deleterious to wild bumblebees (Otti & Schmid-Hempel 2008, Rutrecht & Schmid-Hempel 2008, Cameron *et al*. 2011, Cameron *et al*. 2016) and therefore understanding the impact of this parasite may, currently, be of greater importance. However, to date no quantifiable inoculation protocol has been developed for *N. bombi* (for the most developed methodology see, Rutrecht & Brown 2008). Here, I have developed a novel protocol for individual, quantifiable, inoculations of developing *B. terrestris* larvae. Inoculating each larvae with 50,000 *N. bombi* spores resulted in a 42 – 51 % infection success rate in control bees, post eclosure. However, I recognise that these data are based on an inoculum created from a single infected *B. terrestris* queen, and that this infection may have contained single or multiple strains of *N. bombi*. Therefore, the infection success rate reported from this methodology may differ when testing on different bumblebee species and when using different parasite strains (Schmid-Hempel & Loosli 1998, Schmid-Hempel *et al*. 1999, Carius *et al*. 2001, Rutrecht *et al*. 2007). However, I did identify uniformity in infection success across all donor colonies, suggesting that the methodology is both robust and replicable.

Previous work has shown that phytochemicals can be identified in pollen and nectar of flowering plants (Adler 2000, Stevenson *et al*. 2017) and that these may

impact bumblebee health by reducing *C. bombi* parasite loads *in vivo* (Manson *et al*. 2010, Baracchi *et al*. 2015, Richardson *et al*. 2015). My data shows, for the first time, that phytochemicals found in nectar and pollen can also impact the development of *N. bombi*. In the larval prophylactic bioassay, *N. bombi* infection intensities in newly eclosed workers were significantly lower when compared to a control group. In contrast there was a trend for lower infection intensities in the therapeutic bioassay, however no significant relationship was identified. In addition, there is no evidence from either larval bioassay that *N. bombi* development is completely inhibited in the presence of biochanin A. The potential antifungal activity of biochanin A has been suggested to be due to its planar structure and methoxyl group location, which can compete for fungal cell wall receptor sites (Weidenbörner *et al*. 1990, Rojas *et al*. 2006). This suggests that continuous exposure may lead to an increased concentration of biochanin A inside developing bumblebee larvae, which may in turn be suboptimal for *N. bombi* development and subsequent infection success. It is likely then, that biochanin A is impacting geminating spores by interfering with cellular membrane function, whilst inside the gut of the developing bumblebee larvae, and that biochanin A at my tested concentration has not completely saturated fungal cell wall receptor sites. Given that biochanin A is present in the gut of the prophylactic larvae at the point of inoculation it is likely that *N. bombi* spores entering the gut are immediately exposed to suboptimal conditions and this may explain why the prophylactic treatment had a significant effect when compared to the therapeutic treatment.

When infected adult workers were continuously exposed to biochanin A there was a significant reduction in the recorded infection intensity when compared to the control group at day three, five and seven. Interestingly, biochanin A had no significant therapeutic effect in developing larvae, which appears to be in contrast to my data for infected workers. Given that the physiology of workers and larvae are different (Wilson 1971) it is likely that *N. bombi* has evolved separate developmental strategies. The parasite may switch from an infective phase to a germinating phase following host eclosure (Rutrecht & Brown 2008). Consequently a transition in *N. bombi* infection dynamics may result in the significant therapeutic effect that was observed in adult *B. terrestris* workers, which was not recorded in larvae. Finally biochanin A had no effect on the proportion of bumblebees that eclosed with *N. bombi* infections across all of my bioassays. Therefore biochanin A at the concentrations I exposed larvae to, which were at the high end of natural levels, is

unable to completely inhibit *N. bombi* infection during larval development, suggesting that microsporidian cell wall receptor sites are not completely saturated, even during continuous exposure to the compound. However, my results do outline that bumblebees foraging on species such as red clover, which may have beneficial phytochemistry, may mitigate the impact of disease.

Emerging fungal diseases have been linked to population collapses in a range of fauna, most notably white-nose syndrome in bats and the global spread of chytrid in amphibians (Berger *et al*. 1998, Leopardi *et al*. 2011). Unfortunately, the negative impact of emerging fungal diseases can also be seen in bumblebees (Cameron *et al*. 2011, Cameron *et al*. 2016). The global transportation of commercial bumblebees has exposed naïve bees to novel parasites that have impacted both geographic range and population density of the affected species (Cameron *et al*. 2011). Consequently the environmental prevalence of *N. bombi* is rising (Cameron *et al*. 2016). Infection with this parasite has a number of detrimental impacts on its bumblebee host (Shykoff & Schmid-Hempel 1991, Otti & Schmid-Hempel 2008, Rutrecht & Brown 2009). I believe the novel, quantifiable, inoculation methodology (described above) will enable researchers to use *N. bombi* to answer pertinent evolutionary ecology questions using a host-parasite system with high virulence. In addition, naturally occurring phytochemicals may impact *N. bombi* development in different life stages of its bumblebee host, thus providing positive health benefits. Consequently investigating ecologically relevant phytochemicals may help ameliorate the impact of emerging pollinator diseases.

Chapter 5

The effect of two phytochemicals found in the floral rewards of UK based Agri-environment scheme plants on the bumblebee parasite *Nosema bombi*

Abstract

Some species of bumblebee are undergoing both range and population declines, and this is due in part to a reduction in wildflower meadows and an increase in parasite prevalence. To mitigate the impact of depauperate floral resources in agricultural landscapes, Agri-environment schemes (AES) have been developed and have been shown to be effective in increasing both pollinator abundance and fitness. However, pollen and nectar also contain phytochemicals, which can have a range of impacts on pollinators. Interestingly, some phytochemicals have antimicrobial properties and may indirectly benefit pollinators by reducing parasite loads, potentially increasing the value of AES. Here I test the therapeutic and prophylactic bioactivity of the phytochemicals caffeine and tricoumaroyl spermidine, which were isolated from the floral rewards of AES plants, against the bumblebee parasite *Nosema bombi in vivo*. Caffeine reduced the infection intensity of *N. bombi* in adults that were inoculated as larvae in both the therapeutic and prophylactic bioassays. My results suggest that planting strategies that benefit pollinators, such as AES, could be tailored to provide indirect fitness benefits to pollinators by selecting plants with beneficial phytochemistry.

Key words; *Bombus terrestris*, pathogen, microsporidia, caffeine, tricoumaroyl spermidine, pollinator health, *Lotus corniculatus*, *Onobrychis viciifolia*

5.1 Introduction

Global biodiversity may currently be undergoing a sixth mass extinction event (Barnosky *et al*. 2011). A number of factors have been attributed to this including a reduction in natural heterogeneous habitat, due in part to an increase in intensive agriculture (Krebs *et al*. 1999, Donald *et al*. 2001, Ollerton *et al*. 2014), and an increase in disease prevalence, including a rise in emerging infectious diseases (EIDs) (Daszak *et al*. 2000, Jones *et al*. 2008). The consequences of global biodiversity loss are well documented (Chapin *et al*. 2000), especially so for insect pollinators (Biesmeijer *et al*. 2006, Potts *et al*. 2010). Insect pollinators, such as bumblebees, are critical in maintaining global food production (Breeze *et al*. 2011, Tilman *et al*. 2011, Kleijn *et al*. 2015). However, some species of bumblebee are undergoing population and range declines (e.g., Williams 1982 Fitzpatrick *et al*. 2007, Colla & Packer 2008, Cameron *et al*. 2011) and these in part are a consequence of both intensive agriculture (Carvell *et al*. 2006) and disease prevalence (Cameron *et al*. 2011).

Bumblebees are relatively simple eusocial insects, which form small, annual colonies initiated by a single queen (Sladen 1912, Wilson 1971, Alford 1978). During the lifecycle of a colony, bumblebees are exposed to a range of microbial pathogens (Schmid-Hempel 1998), some of which have been linked to population declines (Cameron *et al*. 2016) and a reduction in colony fitness (Brown *et al*. 2003). In addition, bumblebees are vulnerable to EIDs (Fürst *et al*. 2014), including emerging fungal diseases (Cameron *et al*. 2016, Brown 2017). A microsporidian pathogen of bumblebees that has relatively low prevalence (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014) but has major negative effects on its host (Otti & Schmid-Hempel 2008, Rutrecht & Brown 2009) is *Nosema bombi* (Fantham & Porter 1914). This parasite has been linked to the range and population declines in some naïve North American bumblebee species (Cameron *et al*. 2011, Cameron *et al*. 2016), and this pattern of high virulence in naïve hosts can be seen in other fauna that are impacted by emerging fungal diseases (Berger *et al*. 1998, Leopardi *et al*. 2011). Consequently, mitigating the impact of highly virulent pollinator pathogens such as *N. bombi* is of critical importance. Interestingly, for other insect pollinators a diverse suite of forage plants has been shown to be beneficial for both health and immunity against diseases (Alaux *et al*. 2010).

Pollinators such as bumblebees require a diverse range of flowers to ensure their nutritional needs are met (Vaudo *et al*. 2016). However, an increase in the land set aside for intensive agriculture has led to a depauperate assemblage of wildflowers on which bumblebees can forage (Carvell *et al*. 2006), and this in turn has contributed to wild bumblebee declines (Ollerton *et al*. 2014). Strategies have been developed that attempt to replenish the substantial loss of wildflowers as a consequence of intensive agriculture such as the European Agri-environment schemes (AES) (EU Common Agricultural Policy 2015) and US based Conservation Reserve Programs (USDA Farm Service Agency 2016). These schemes contain prescriptions that increase floral abundance and diversity (Natural England 2017), which have, in turn, been shown to increase insect pollinator abundance and fitness (Pywell *et al*. 2006, Carvell *et al*. 2007, Wood *et al*. 2015, Carvell *et al*. 2017). Interestingly, pollen and nectar contain secondary metabolites (Baker & Baker 1975, Adler 2000, Stevenson *et al*. 2017). Some secondary metabolites have antimicrobial activity and may indirectly improve bumblebee health by reducing parasite loads (Manson *et al*. 2010, Richardson *et al*. 2015, Giacomini *et al*. 2018). To date this area of research has been limited to the impact of phytochemicals on the prevalent bumblebee trypanosome parasite *Crithidia bombi* (Gorbunov 1987) both *in vivo* (Manson *et al*. 2010, Richardson *et al*. 2015) and *in vitro* (Palmer-Young *et al*. 2016, Palmer-Young & Thursfield 2017). Whilst this parasite exhibits condition dependent virulence (Brown *et al*. 2000), and has significant impacts on colony productivity (Brown *et al*. 2003) it may not be as deleterious as *N. bombi* to wild bumblebee populations (Cameron *et al*. 2011, Cameron *et al*. 2016). This poses the question of whether phytochemicals found in floral rewards may have a similar effect on microsporidian infections and, if they do, whether manipulation of floral resources in initiatives such as AES could minimize the impact of *N. bombi* on wild bumblebee populations.

The development of a novel inoculation protocol (this thesis 4.2.3) has enabled the design of bioassays that can investigate the effect of phytochemicals on *N. bombi*. Here, I inoculate *Bombus terrestris* larvae with *N. bombi* and test two phytochemicals (caffeine and tricoumaroyl spermidine) recovered from the pollen and nectar of AES plants at their ecologically relevant concentrations to answer the following questions. (1) Does larval prophylactic treatment with caffeine or tricoumaroyl spermidine reduce *N. bombi* infection intensity in eclosed adult

workers? and (2) Does larval therapeutic treatment with caffeine or tricoumaroyl spermidine reduce *N. bombi* infection intensity in eclosed adult workers?

5.2 Methods

5.2.1 Chemical analysis of AES plants

For methods describing the chemical analysis of the pollen and nectar of AES plants please refer to section 3.2.1 of this thesis. The alkaloid caffeine was identified in the nectar of sainfoin (*Onobrychis viciifolia*) using an rt of 6.11 and an *m/z* of 195.09 and the polyamide tricoumaroyl spermidine was identified in the pollen of bird's-foot trefoil (*Lotus corniculatus*) using an rt of 13.85 and an *m/z* of 584.27. Caffeine and tricoumaroyl spermidine have previously reported biological activity against microbes (Richardson *et al*. 2015, Walters *et al*. 2001, respectively) and therefore were ideal AES phytochemicals to test for biological activity, at ecologically relevant concentrations, against *N. bombi*. Both phytochemicals were assigned a concentration in parts per million (ppm) using UV absorption as a measurement criterion (caffeine = 39ppm, TCS = 208ppm as recovered in this thesis 3.3.1.). This was calculated using an available standard at values chosen to give a realistic floral reward concentration range (10, 100, 500, 1000 ppm) (Adler 2000, Cook *et al*. 2013). If there was no standard available for a phytochemical then an available compound with a similar UV absorption, based on chromophore orientation, was selected to provide a close approximation.

5.2.2 Colony provenance

Eight *Bombus terrestris audax* colonies (hereafter referred to as donor colonies) (containing a queen, brood and a mean of 45 (± 6.5 S.E.) workers) were obtained from Biobest, Belgium. Colonies were kept in a dark room at 26°C and 50% humidity (red light was used for any colony manipulation). To ensure colonies were healthy and developing normally they were monitored for 7 days prior to use in any experimental procedures. This included randomly screening 10% of the workers every two days, from each colony, for parasitic infections in faeces using a phase-contrast

microscope set to ×400 magnification. No infections were identified in any of the eight donor colonies.

Micro-colonies were established by removing 8 patches of brood containing approximately 10 developing larvae (growth stage L2-3), from each of the eight donor colonies. Each of these patches of brood were placed in an individual 14×8×5.5cm acrylic box. The micro-colonies were each provisioned with *ad libitum* pollen and sugar water, and 3 workers from their original donor colony to provide brood care. All pollen used throughout the experiment was irradiated to remove any microbes. Prior to being entered into the experiment all brood-caring workers were individually marked using a coloured, numbered Opalith tag and recorded.

5.2.3 Artificial inoculation of *B. terrestris* larvae with *N. bombi*

For methods describing the artificial inoculation of *B. terrestris* larvae with *N. bombi* please refer to section 4.2.3 of this thesis.

5.2.4 Do phytochemicals impact *N. bombi* infection in developing *B. terrestris* larvae?

To test for any prophylactic effect of caffeine or tricoumaroyl spermidine, 32 micro-colonies (4 per donor colony) as described above were used. Prior to inoculation, control larvae were kept in their original micro-colonies (n=16, 8 per treatment) and provided *ad libitum* pollen and sugar water. However, in the experimental groups *ad libitum* pollen and sugar water containing either caffeine at 39 ppm (n=8 micro-colonies) or tricoumaroyl spermidine at 208 ppm (n=8 micro-colonies) was provided for 7 days.. Phytochemicals were added to sugar water using 4ml of 40% MeOH as a solvent per litre, control colonies also had 4ml of 40% MeOH added per litre of sugar water. After seven days both experimental and control larvae were artificially inoculated with 50,000 *N. bombi* spores in 4.3µl of inoculant using a 20µl pipette (as described in section 4.2.3 of this thesis). The spore concentration in the inoculum is within ecologically relevant values for *N. bombi* spores in faeces and has been shown to be a concentration that is infective to developing *Bombus terrestris* brood (Rutrecht

& Brown 2008, this thesis 4.3.1).The larvae were left to consume the inoculum for 30 minutes, before being returned to their micro-colony.

In a simultaneous therapeutic effect of caffeine and tricoumaroyl spermidine, 32 micro-colonies (4 per donor colony) as described above were used. For each phytochemical treatment larvae were each inoculated with 50,000 *N. bombi* spores in 4.3µl of experimental inoculant, as described above, using a 20µl pipette. The larvae were left for 30 minutes to consume the inoculum. The inoculated larvae were returned to their respective micro-colonies with brood-caring workers. Each control micro-colony (n=16, 8 per phytochemical treatment) was provisioned with *ad libitum* pollen and sugar water. However, in the experimental groups *ad libitum* pollen and sugar water containing either caffeine (Sigma Aldrich CO750) at 39 ppm (n=8 micro-colonies) or tricoumaroyl spermidine (produced via synthesis, Appendix 9.1) at 208 ppm (n= 8 micro-colonies) was provided for 7 days. As before, phytochemicals were added to sugar water using 4ml of 40% MeOH as a solvent per litre, control colonies also had 4ml of 40% MeOH added per litre of sugar water

In both feeding trials larvae were allowed to develop naturally and pupate in their respective micro-colonies. Once eclosed, new workers were marked using a coloured, numbered Opalith tag and individually quarantined for 3 days in an inverted plastic cup, which was modified with a hole that enabled a 15ml falcon tube to be inserted. The falcon tube contained control inverted sugar water diluted with double distilled H_2O (50% w/w) that the newly eclosed workers could feed on. A quarantine period of three days was used to ensure that faecal samples were not heavily contaminated with pollen, as this can complicate parasite quantification. At the end of the quarantine period each worker had its thorax measured (mm) as a proxy for bumblebee size, using a set of Mitutoyo^{tm} digital calipers. In addition, each worker was isolated in a 25ml plastic vial where it provided a faecal sample, collected in a 10 µl glass capillary, the volume (µl) of which was recorded. Following this, each worker's faecal sample was screened for *N. bombi* by microscopic examination using a phase-shift microscope at ×400 magnification. If an infection was identified a Neubauer improved haemocytometer was used to quantify the parasite load. Workers were then sacrificed and stored in a labeled Eppendorf tube at -80°C.

5.2.5 Statistical analysis

All statistical analyses and graphical outputs were undertaken in R open source programming language (R Core Team 2018, Wickham 2009). To analyse the therapeutic and prophylactic effect of caffeine and tricoumaroyl spermidine on *N. bombi* infection intensity in newly eclosed workers, two separate linear mixed-effects models (LMM) were constructed. Models were constructed in the R package 'lme4' (Bates *et al*. 2015). Both models were constructed under the following parameters. Infection intensity was used as a response variable, with treatment group, thorax width (mm) and faeces volume (µl) as designated covariates. Both models also incorporated donor colony as a random effect. Models were validated in R by visually checking the normality of residuals, and for overdispersion and collinearity of variables.

5.3 Results

5.3.1 Do phytochemicals in AES plants have a prophylactic effect on *N. bombi* infection intensity?

In the caffeine prophylactic bioassay, 82 workers successfully eclosed of which 36 had *N. bombi* infections (control = 22, caffeine = 14), resulting in an overall infection success of 44%. In the tricoumaroyl spermidine prophylactic bioassay, 72 workers successfully eclosed, of which 31 had *N. bombi* infections (control = 16, tricoumaroyl spermidine = 15) leading to an overall infection success of 43%. There were no significant differences between the infection prevalence in either phytochemical treatment (caffeine $\chi^2 = 1.433$, $P = 0.231$, tricoumaroyl spermidine $\chi^2 = 0.196$, $P = 0.658$) (Figure 5.1)

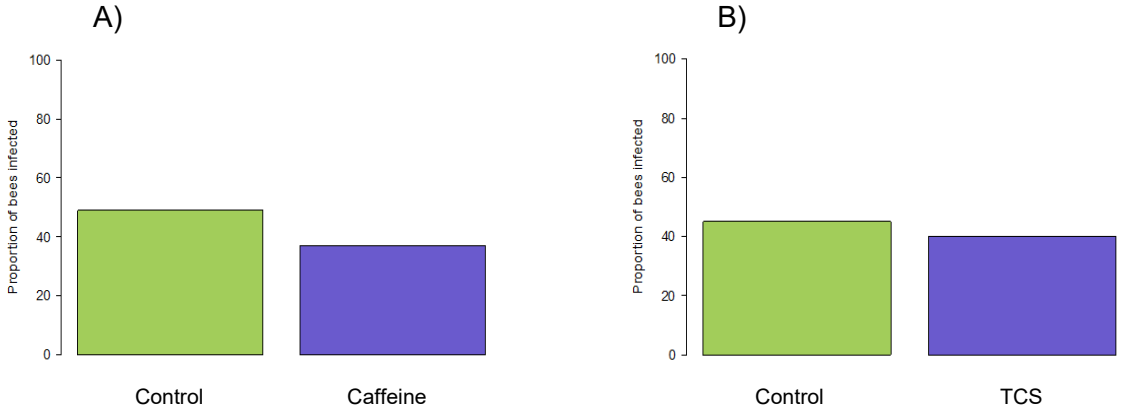


Figure 5.1 *Nosema bombi* infection success in adult *B. terrestris* workers in both the caffeine (A) and tricoumaroyl spermidine (TCS) (B) prophylactic bioassays. There was no significant difference between infection success in either treatment (caffeine $\chi^2 = 1.433$, $P = 0.231$, tricoumaroyl spermidine $\chi^2 = 0.196$, $P = 0.658$).

Caffeine had a significant prophylactic effect by reducing *N. bombi* infection intensity in eclosed workers (LMM, $F_{1,28} = 15.33$, $P < 0.001$). The covariates thorax width (LMM, $F_{1,28} = 3.75$, $P = 0.06$), faeces volume (LMM, $F_{1,28} = 0.65$, $P = 0.4$) and the random effect colony ($P = 0.27$) all had no significant effect on *N. bombi* infection intensity. In contrast, tricoumaroyl spermidine had no significant prophylactic effect on *N. bombi* infection intensity when compared to a control group (LMM, $F_{1,26} =$

0.514, P = 0.479). Similarly to the caffeine treatment, the covariates thorax width (LMM, $F_{1,26}$ = 0.61, P = 0.44), faeces volume (LMM, $F_{1,26}$ = 1.49, P = 0.23), and the random effect of colony (P = 0.9) also had no significant effect on *N. bombi* infection intensity (Figure 5.2).

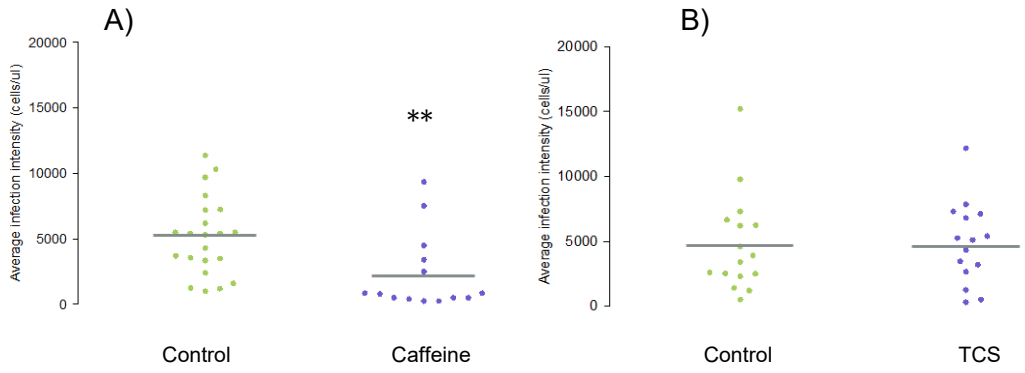


Figure 5.2 Beeswarm plot, used to show the complete spread of data, of *N. bombi* infection intensities in adult *B. terrestris* workers for both the caffeine ((A), n = 36) and tricoumaroyl spermidine (TCS) ((B), n = 31) prophylactic bioassays. The sample mean has been marked with a grey bar and statistical differences have been marked with a double asterisk. Caffeine had a significant prophylactic effect on *N. bombi* infection intensity (LMM, $F_{1,28}$ = 15.33, P < 0.001).

5.3.2 Do phytochemicals in AES plants have a therapeutic effect on *N. bombi* infection intensity?

In the caffeine therapeutic bioassay, 101 workers successfully eclosed, of which 39 had *N. bombi* infections (control = 25, caffeine = 14), resulting in an overall infection success of 39%. In the tricoumaroyl spermidine therapeutic bioassay, 67 workers successfully eclosed, of which 29 had *N. bombi* infections (control = 15, tricoumaroyl spermidine = 14) leading to an overall infection success of 43%. There were no significant differences between the infection prevalence in either phytochemical treatment (caffeine $\chi^2 = 2.384$, $P = 0.123$, Tricoumaroyl spermidine $\chi^2 = 0.005$, $P = 0.941$) (Figure 5.3).

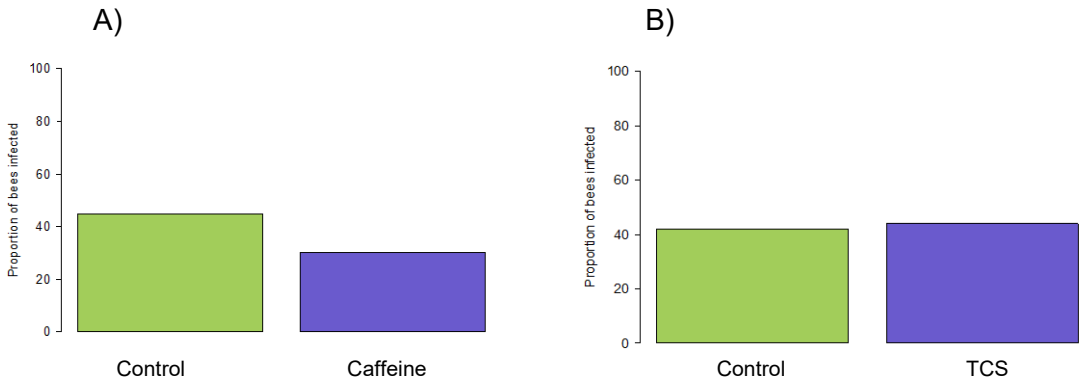


Figure 5.3 *Nosema bombi* infection success in adult *B. terrestris* workers in both the caffeine (A) and tricoumaroyl spermidine (TCS) (B) therapeutic bioassays. There was no significant difference between infection success in either treatment (caffeine $\chi^2 = 2.384$, $P = 0.123$, tricoumaroyl spermidine $\chi^2 = 0.005$, $P = 0.941$).

The phytochemical caffeine had a significant therapeutic effect by reducing *N. bombi* infection intensity in eclosed workers (LMM, $F_{1,31} = 4.97$, $P = 0.032$). The covariates thorax width (LMM, $F_{1,31} = 2.49$, $P = 0.123$), faeces volume (LMM, $F_{1,31} = 1.87$, $P = 0.181$), and the random effect colony ($P = 0.193$) all had no significant effect on *N. bombi* infection intensity. In contrast, tricoumaroyl spermidine had no significant therapeutic effect on *N. bombi* infection intensity when compared to a control group (LMM, $F_{1,24} = 0.516$, $P = 0.48$). As with the caffeine experiment, the covariates thorax width (LMM, $F_{1,24} = 1.99$, $P = 0.171$), faeces volume (LMM, $F_{1,24} = 1.14$, $P =$

0.29), and the random effect colony (P = 0.9) again had no significant effect on *N. bombi* infection intensity (Figure 5.4).

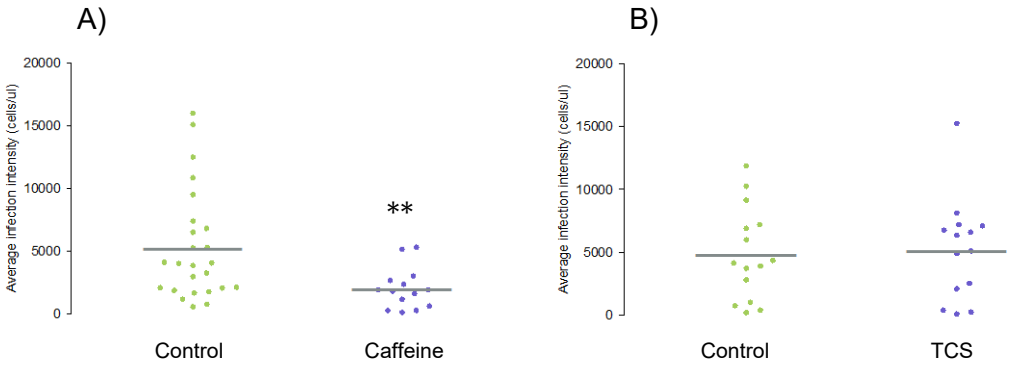


Figure 5.4 Beeswarm plot, used to show the complete spread of data, of *N. bombi* infection intensities in adult *B. terrestris* workers for both the caffeine ((A), n = 39) and tricoumaroyl spermidine (TCS) ((B), n = 29) therapeutic bioassays. The sample mean has been marked with a grey bar and statistical differences have been marked with a double asterisk. Caffeine was found to have a significant therapeutic effect on *N. bombi* infection intensity (LMM, $F_{1,31}$ = 4.97, P = 0.032).

5.4 Discussion

My results show for the first time that phytochemicals identified in AES plants at their ecologically relevant concentration can impact microsporidian infections in the important bumblebee pollinator *B. terrestris*. Caffeine, which was recovered from the nectar of the legume species *O. viciifolia,* at a concentration of 39 ppm, significantly reduced the infection intensity of *N. bombi in vivo,* both prophylactically and therapeutically. Consequently, bioactive phytochemicals found in AES plants may mitigate the impact of emergent pollinator diseases.

Secondary metabolites are synthesized by plants and are defined as any compounds that are not used in primary growth (Schoonhoven *et al*. 2005). These compounds, which may have bioactive properties (Cowan 1999) can translocate into

floral rewards where they may be consumed by pollinators (Adler 2000). In chapter three of this thesis I identified the methlxanthine caffeine in the nectar of *O. viciifolia* and the polyamide tricoumaroyl spermidine in the pollen of *L. corniculatus*. These herbaceous angiosperm species are both included in AES planting strategies (Natural England 2017) and are likely to be visited by foraging bumblebees in AES enhanced landscapes. Caffeine has been reported in thirteen orders of plants, with evidence to suggest that it has evolved independently five times (Huang *et al*. 2016). Consequently, caffeine likely has a strong biological value for angiosperms, either as a herbivore deterrent (Bernays *et al*. 2000) or as a pollinator manipulator (Wright *et al*. 2013). Tricoumaroyl spermidine is a common constituent of plant pollen (Strack *et al*. 1990) and may function as a phagostimulant (Lin & Mullin 1999). Interestingly, both of these phytochemicals have known biological activity (caffeine, Raj & Dhala 1965; tricoumaroyl spermidine, Walters *et al*. 2001) and may therefore impact pollinator health. AES floral seed mixes have been refined to increase pollen and nectar resources for pollinators in intensely farmed landscapes (Pywell *et al*. 2011 (a)). However, the impact of the diverse biochemistry generated by these schemes is often overlooked (Stevenson *et al*. 2017). Consequently understanding the ecological significance of phytochemicals in floral rewards and how they interact with pollinators is critical in evaluating the effectiveness of AES.

Bumblebee declines, as outlined above, have been linked to an increase in parasite prevalence (Cameron *et al*. 2011, Cameron *et al*. 2016). Parasite infection can significantly reduce host fitness (Hudson *et al*. 1992) and EIDs as a result of infection can lead to substantial declines in host populations (Berger *et al*. 1998, Cameron *et al*. 2011, Leopardi *et al*. 2011). My results show that consumption of ecologically relevant levels of caffeine in nectar can significantly reduce *N. bombi* infection intensity in adult *B. terrestris* workers, which were inoculated as larvae. This novel relationship was identified in both prophylactic and therapeutic treatments. Both of these treatment methods are recognized in preventative medicine and are biologically realistic within the bumblebee - *Nosema*, host - parasite system. Infection with *N. bombi* is thought to occur between conspecifics at communal areas (van der Steen 2008) and the likelihood of infection is not restricted to a singular time point (Jones & Brown 2014). Consequently *N. bombi* infection may occur during the anthesis of AES flowers and may therefore interact with prophylactic or therapeutic treatment. Within the bioassays designed for this investigation exposure to caffeine occurred over a

period of seven days. However, as anthesis in *O. viciifolia* occurs over a longer period of time, bumblebees may be exposed to the floral chemistry of such species for a longer duration. Of course, under natural settings, bumblebee colonies are likely exposed to a diverse suite of phytochemicals (Adler 2000, Stevenson *et al*. 2017), and these may have varying impacts on bumblebees (Wright *et al*. 2013, Arnold *et al*. 2014, Richardson *et al*. 2015), not identified in this study. Whilst there was no significant reduction in the proportion of bumblebees that eclosed with infection across both caffeine bioassays, there was a trend for lower infection prevalence when compared to the control group. Caffeine is well known for its biological activity (Raj & Dhala 1965), and it is likely that caffeine has a negative effect on *N. bombi* spore germination and subsequent infection success in developing larvae. Bumblebee larvae have a blind gut (Wilson 1971) and therefore all nutrients are either directly absorbed by the developing larvae for growth or they remain in the gut indefinitely. Consequently, continuous exposure to caffeine, either prophylactically or therapeutically, may lead to an increase in caffeine concentration in the larval gut, which may, in turn, be unsuitable for *N. bombi* development and propagation. In contrast to caffeine, tricoumaroyl spermidine, in both the therapeutic and prophylactic bioassays, had no significant impact on *N. bombi* prevalence or infection intensity when compared to a control group. In previous work (this thesis 3.2.1) tricoumaroyl spermidine at ecologically relevant concentrations had no impact on the growth of the bumblebee trypanosome parasite *C. bombi* and it is likely that as a common pollen constituent (Strack *et al*. 1990) found at relatively high concentrations (this thesis 3.3.1), bumblebee parasites may have evolved resistance to any potential biological activity (Palmer-Young *et al*. 2016).

One of the functions of AES is to increase floral abundance and diversity in agricultural landscapes (Natural England 2017). Here I have shown that these schemes also expose pollinators to bioactive phytochemicals. However, the chemical composition and concentration of floral rewards can vary geographically (Tiedeken *et al*. 2016) and in response to domestication (Egan *et al*. 2018). Consequently, in different AES landscapes bumblebees may encounter the same genus of flowering plant with different chemical profiles. Nevertheless, I would note that my samples (this thesis 3.3.1) have been pooled from multiple flowers across multiple locations within the UK and therefore are a realistic representation of chemical diversity within a given genus. Consequently my results provide the first robust investigation into how

ecologically relevant concentrations of phytochemicals can impact microsporidia infections in bumblebees.

Previous AES seed mixes have been developed dynamically with the scientific community (Pywell *et al*. 2011, Dicks *et al*. 2015) and these recommendations were largely based on visitation and abundance records. Whilst florally enriched landscapes have been shown to provide direct fitness benefits to bumblebees (Carvell *et al*. 2017) I suggest that dynamic schemes such as AES could be further tailored to include or increase the abundance of plants that are known to provide indirect fitness benefits to pollinators via bioactive phytochemicals.

Bumblebee populations are under threat due in part to a reduction in wildflower meadows and an increase in parasite prevalence. AES not only increase floral abundance and diversity in agricultural landscapes but may also expose pollinators to a diverse suite of phytochemicals, some of which may have biological activity. Consequently, determining the chemical profile of forage plants is key in understanding how landscapes and schemes such as AES can impact on pollinator diseases. My results suggest that initiatives such as AES could be tailored to provide indirect fitness benefits to pollinators by providing beneficial phytochemicals that may reduce parasite loads.

Chapter 6

The effect of the phytochemical caffeine on the epidemiology of *Nosema bombi*, a microsporidian parasite of bumblebees

Abstract

Emergent infectious disease epidemics have a negative impact on the fitness of their host. Epidemiological studies can help elucidate the incidence and distribution of these diseases and enable the design of effective intervention strategies. A shift from solitary to group living is believed to be one of the major transitions in evolution. Nevertheless social groups are arguably more susceptible to diseases. For example social insects, which provide economically important ecosystem services, are susceptible to a range of diseases throughout the colony lifecycle. More specifically, infection with the microsporidian *Nosema bombi* has been linked to population and range declines in a number of North American bumblebees. Previous work has shown that phytochemicals found in pollen and nectar can negatively impact on bumblebee parasites either *in vitro* or *in vivo*, but only in individual animals, which ignores the social aspects of their epidemiology. Here I attempt to address this evidence gap by investigating the impact of the methylxanthine caffeine, found in the nectar of sainfoin (*Onobrychis viciifolia*), on the epidemiology of *N. bombi* in wild caught and reared *Bombus terrestris* colonies. Caffeine reduced both the prevalence and infection intensity of *N. bombi* in *B. terrestris* colonies when compared to a control group. In addition my results suggest that a bloom in *N. bombi* parasitaemia in young bumblebees is linked to higher intracolonial infection prevalence. Chronic exposure to caffeine did not significantly reduce the population densities or the quantity of sexual castes produced by *N. bombi* infected colonies, suggesting that they are not paying a cost for the protective benefits of this chemical, at least under laboratory conditions. Ultimately my results suggest that schemes that increase floral abundance to support biodiversity can also be used as disease management tools as they may negatively impact the social epidemiology of important pollinator diseases.

Key words; *Bombus terrestris*, pathogen, secondary metabolites, emergent infectious diseases, Agri environment schemes, pollinator health, sainfoin, *Onobrychis viciifolia,* disease management.

6.1 Introduction

Infectious disease epidemics can negatively impact both the population density and fitness of a host organism (Hudson *et al*. 1992, Berger *et al*. 1998). Consequently, epidemiological studies are critically important in understanding the incidence and distribution of diseases (Cox & Subbarao 2000, Keeling *et al*. 2001), which, in turn, may facilitate the design of robust, effective interventions (Ferguson *et al*. 2001, Longini Jr. *et al*. 2005). In addition fine-scale, population level studies can guide policymakers on the best practice to minimise the impact of disease epidemics (Riley *et al*. 2003, Haydon *et al*. 2006, Rambaut *et al*. 2008). Such studies are particularly important because incidences of disease epidemics, including emerging infectious diseases (EIDs), are increasing globally (Jones *et al*. 2008). A switch from solitary to social living is thought to be one of the major evolutionary transitions (Maynard Smith & Szathmary 1995) and living in groups may incur individual fitness benefits (Hamilton 1964 (a) & (b)). However, social groups may be more susceptible to diseases (Anderson & May 1979, Pedersen *et al*. 2005) and epidemiology in social systems is arguably more complex than in solitary organisms (Andrewes 1964). Social organisms may facilitate the spread of infectious diseases due to their relatively high population densities and regular contact with conspecifics (Riley *et al*. 2003, Haydon *et al*. 2006, Otterstatter & Thomson 2007). Consequently understanding the epidemiology of diseases that affect social organisms may improve our understanding of host-parasite dynamics in social systems more generally and help to improve disease intervention strategies.

Social insects provide an excellent model system to investigate the impact of diseases on social systems. Social insects are susceptible to a range of diseases throughout the life cycle of a colony (Schmid-Hempel 1998), and in addition to individual immunity, social insects have also evolved social immune mechanisms (termed 'social immunity' reviewed in Cremer *et al*. 2007). Social immunity may limit the spread of diseases throughout a colony, via structured contact networks (Naug & Camazine 2002, Naug 2008) or destructive disinfection of brood (Pull *et al*. 2018), for example. Nevertheless, these mechanisms do not fully protect the developing colony, especially from EIDs (Genersch *et al*. 2006, de Miranda & Genersch 2010, Fürst *et al*. 2014). In addition to their value as model systems, social insects also provide economically important ecosystem services including nutrient

redistribution (Griffiths *et al*. 2017), biomass decomposition (Collins 1981) and pollination (Breeze *et al*. 2011, Kleijn *et al*. 2015). Consequently, understanding the diseases of social insects is of vital importance to ensure that these essential ecosystem services are maintained.

Bumblebees form part of an economically important pollinator network (Breeze *et al*. 2011, Kleijn *et al*. 2015). These charismatic pollinators are susceptible to infection from a range of microbial pathogens (Schmid-Hempel 1998), which may reduce overall colony fitness (e.g., Brown *et al*. 2003). Due to the high-density of individuals found within a bumblebee colony, interactions between closely related individuals and different developmental stages are common (Otterstatter & Thomson 2007, Folly *et al*. 2017). In addition, bumblebees regularly come into contact with hetero-colonial conspecifics and congeners at shared floral resources (Durrer & Schmid-Hempel 1994, Ruiz-González *et al*. 2012). Consequently, bumblebees may be effective intra- and interspecific vectors of disease (Meeus *et al*. 2011, Fürst *et al*. 2014). One such parasite that may be transmitted among and within bumblebee colonies is the microsporidian *Nosema bombi* (Fantham & Porter 1914). This pathogen is presumably transmitted by shared contact between individuals (van der Steen 2008) and has a relatively low environmental prevalence in Europe (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014). Arguably *N. Bombi* has higher virulence when compared with other prevalent bumblebee parasites (Otti & Schmid-Hempel 2008) and critically, infection is thought to be a driver of population and range declines in some North American bumblebees (Cameron *et al*. 2011). Consequently, understanding its social epidemiology may be critical for disease management strategies.

Bumblebees are unable to recognise and remove *N. bombi* infected brood (Munday & Brown 2018). Consequently, bumblebees may not have a sufficient social immune repertoire to adequately prevent disease transmission within a colony as has been shown in other social insects (Pull *et al*. 2018). Interestingly, the infection intensity of *Crithidia bombi* (Gorbunov 1987), a bumblebee parasite with higher prevalence (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014) than *N. bombi*, has been reduced *in vivo* when bumblebees consumed phytochemicals in sugar water (Manson *et al*. 2010, Richardson *et al*. 2015). More recently sunflower (*Helianthus annuus*) pollen significantly reduced the infection intensity of *C. bombi* in *B. impatiens* workers and *N. ceranae* in *Apis mellifera* workers (Giacomini *et al*. 2018).

Infection intensity is linked to infection success and ultimately prevalence in bumblebee parasites (Ruiz-González & Brown 2006). Consequently beneficial floral compounds that reduce infection intensity may in turn reduce parasite environmental prevalence and impact on disease epidemiology.

Pollen and nectar contain phytochemicals (Baker & Baker 1975, Adler 2000, Stevenson *et al*. 2017) and these may be consumed by foraging bumblebees. Some of these compounds can have negative impacts on pollinators (Cook *et al*. 2013, Tiedeken *et al*. 2016). However, some may have antimicrobial activity (Cowan 1999) and may therefore indirectly benefit bumblebees by controlling pathogens and parasites (Manson *et al*. 2010, Richardson *et al*. 2015, this thesis 4.3, 5.3). Agri-environment schemes (AES) and Conservation reserve programs (CRP) are interventions that aim to support biodiversity in areas of intensive agriculture (EU Common Agricultural Policy 2015, USDA Farm Service Agency 2016, Natural England 2017). These schemes increase both the abundance and diversity of flowers (Pywell *et al*. 2011 (a), Dicks *et al*. 2015) to benefit pollinators (Carvell *et al*. 2017). However, these schemes may also indirectly increase the diversity of phytochemicals that pollinators, more specifically bumblebees, are exposed to. Interestingly, the methlxanthine caffeine has been identified in the nectar of common sainfoin (*Onobrychis viciifolia*) (this thesis 3.3.1), a component of agri-environment schemes in the UK, as well as a major global crop (Food and Agriculture Organization of the United Nations 1998), and has known biological activity against microbes (Raj & Dhala 1965), including *N. bombi in vivo* (this thesis 5.3). This raises the question of whether phytochemicals, such as those found in AES and CRP, may impact the epidemiology of an important bumblebee pathogen, and whether initiatives such as AES and CRP could be tailored to mitigate the impact of pollinator diseases. Here I inoculate the incipient brood of wild caught bumblebee (*B. terrestris*) queens with *N. bombi* and rear colonies to ask the following questions, 1) Does consumption of caffeine reduce the overall infection intensity of diseased bumblebees within a colony and how does this map on to colony demographics?, 2) Does caffeine consumption reduce intracolonial *N. bombi* infection prevalence and 3) Does caffeine consumption impact the overall fitness of colonies that are infected with *N. bombi*?

6.2 Methodology

6.2.1 Chemical analysis of Agri-environment scheme plants

For methods describing the chemical analysis of the pollen and nectar of AES plants please refer to section 3.2.1 of this thesis. The alkaloid caffeine was identified in the nectar of sainfoin (*Onobrychis viciifolia*) using an rt of 6.11 and an *m/z* of 195.09. Caffeine has previously reported biological activity against microbes (Raj & Dhala 1965, Richardson *et al*. 2015), including reducing *N. bombi* infection intensity in *B. terrestris* (this thesis 5.3). Therefore caffeine was an ideal compound to investigate the impact of AES phytochemicals on *N. bombi* epidemiology.

6.2.2 Bumblebee colony establishment

Between February and April 2018, 250 wild, foraging *B. terrestris* queens were collected from Windsor Great Park, Surrey, UK (SU992703), using an entomological net. Each queen was isolated into an individual, labeled, 25ml collection vial and chilled in an insulated bag, before being returned to the laboratory. All queens were screened for common bumblebee endoparasites (*Apisystis bombi*, *C. bombi*, *N. bombi* and *Sphaerularia bombi*) via microscopic examination of faeces using a phase contrast microscope at ×400 magnification. Following this initial screen, bumblebee queens were quarantined in an individual 127×67×50mm acrylic box, where they were fed *ad libitum* pollen and sugar water, in a dedicated bumblebee rearing room, which was kept at 26°C and 50% humidity. As in previous chapters all pollen used throughout the experiment was irradiated to remove any microbes. Seven days after the initial parasite screen each queen was rescreened for the common bumblebee endoparasites as above. This seven-day delay has been used to ensure that any incipient infections that were missed during the initial screen were identified once parasitemia had increased to detectable levels (Logan *et al*. 2005). All queens that were infected during either of the parasite screens (n = 15) were excluded from the experiment and released back to the original field collection site. Uninfected queens (hereafter referred to as queens) were returned to their acrylic box in the dedicated rearing room and entered into the experiment. Unless otherwise stated all colony manipulation was carried out under red light using sterile equipment and workbenches.

All queens were provided with *ad libitum* sugar water from a gravity feeder and a pollen ball for nutrition and to encourage egg-laying. Pollen balls were created by combining pollen and sugar water (50% w/w) in a 20:1 ratio. This mixture was then subdivided into individual 15×15×15mm cubes. Sugar water and pollen balls were changed every seven days or if a queen had spoiled her resources, whichever came first. Queens were left to develop naturally and reproductive output was monitored and recorded. Once the first set of workers had hatched, these were marked using numbered, coloured Opalith tags (tag colour was unique to cohort, not individual or colony) and the incipient brood was inoculated with *N. bombi* and entered into one of two feeding regimes. Any queen that did not lay a clutch of eggs within eight weeks (n = 113) was excluded from the experiment and released back to the original field collection site. Of the 250 queens originally caught, 40 produced an incipient colony, of which 19 lasted for the duration of the experiment.

6.2.3 *Nosema bombi* inoculation

For methods describing the artificial inoculation of *B. terrestris* larvae with *N. bombi* please refer to section 4.2.3 of this thesis.

All larvae in the incipient colonies, irrespective of instar, were individually inoculated with 50,000 spores in 4.3µl of experimental inoculant, as described above, using a 20µl pipette. The larvae were left for 30 minutes to consume the inoculum and the number of inoculated larvae per colony was recorded (Appendix 9.3). The spore concentration in the inoculum is within ecological relevant values for *N. bombi* spores in faeces and has been shown to be a concentration that is infective to developing *B. terrestris* brood (Rutrecht & Brown 2008, this thesis 4.3.1 & 5.3). The inoculated larvae were then returned to their respective colonies. Once larvae were returned the original queen was allowed to re-associate with the brood for 5 minutes prior to the workers being returned. This step reduced the likelihood of the queen rejecting the brood later in the colony lifecycle (AF personal observation). Each control colony (n = 10 colonies) was provisioned with *ad libitum* pollen and sugar water for the duration of the colony lifecycle. However, in the experimental group *ad libitum* pollen and sugar water, containing caffeine (Sigma Aldrich CO750) at 39 ppm (n = 9 colonies) was provided for the duration of the colony life cycle. Caffeine was added

to sugar water using 4ml of 40% MeOH as a solvent per litre, control colonies also had 4ml of 40% MeOH added per litre of sugar water.

6.2.4 Do phytochemicals impact the epidemiology of *N. bombi* in bumblebees?

Following inoculation, each incipient colony (including queen and any hatched workers) were placed into a 290×220×130mm plastic colony box. This habitation box was connected to a separate 290×220×130mm plastic box that was used as a foraging arena. The foraging arena provided *ad libitum* pollen and experimental sugar water (as described above). Pollen and sugar water were changed every seven days, or once the colony had consumed all of the resources, whichever came first. To investigate if caffeine had any effect on *N. bombi* epidemiology a range of colony specific measurements were taken throughout the colony lifecycle. Every two weeks all newly eclosed workers were individually marked using a coloured, high varnish paint, which was unique to that specific brood cohort and not the colony. Marking bumblebees in this way allowed for investigation into colony demographics and how that impacted infection prevalence and intensity. A period of two weeks was used to ensure that larvae that were at instars L3 & L4 during the previous cohort had hatched for the subsequent cohort. Consequently each cohort contained a meaningful representation of a colony's worker production. To investigate the impact of caffeine on infection prevalence and intensity in foragers, which are the route for inter-colony transmission of the parasite, every seven days 10% of the total colony population that were foraging were removed and screened for *N. bombi* infection via microscopic examination of the faeces. Each forager had its brood cohort and colony recorded. If an infection was identified a Neubauer improved haemocytometer was used to calculate the infection intensity (cells/µl). All foragers, irrespective of *N. bombi* infection were then sacrificed in a -80ºC freezer. Finally, when the colony was terminated all remaining bees were removed and isolated in individual, labeled, 25ml collection tubes and screened for *N. bombi* infection as described above. For each bee a record of sex, brood cohort and colony was taken, before they were sacrificed in a -80ºC freezer. To ensure consistency across colonies with respect to termination, I used the following parameters to define the end of a colony. Colony endpoint was defined as either three weeks following the death of the original queen, three weeks

following the eclosure of the first sexual caste or three weeks after the queen last laid a clutch of eggs. These guidelines ensured that all developing brood would reach eclosure by the endpoint, enabling a robust estimation of the complete reproductive output of a colony.

6.2.5 Statistical analysis

All statistical analyses and graphical outputs were conducted in R open source programming language (R Core Team 2018, Wickham 2009). To analyse the prevalence of *N. bombi* infection and the intensity of infection in colonies two separate linear mixed-effects models (LMM) were constructed. Models were constructed in the R package 'lme4' (Bates *et al*. 2015). The *N. bombi* prevalence model was constructed by having infection state (0/1) as a response variable, with phytochemical treatment, number of inoculated brood, number of days since inoculation and brood cohort as designated covariates. Similarly the *N. bombi* infection intensity model was constructed by having infection intensity (cells/µl) as a response variable with phytochemical treatment, number of inoculated brood, number of days since inoculation and brood cohort as designated covariates. In addition this model also included cohort and treatment as an interaction. Both models also incorporated original colony and forager (0/1) as random effects. In addition to the analysis of infection prevalence and intensity two further models were constructed to investigate impact of treatment on colony fitness. To analyse colony size, worker production since inoculation was used as a response variable with treatment, days since inoculation and number of brood inoculated as designated covariates. In addition, a final model was constructed to analyse the production of reproductive castes. Here sexual production was used as a response variable with treatment, number of brood inoculated, number of days since inoculation and population size at colony terminus as designated covariates. Again, both models included colony as a random effect. Models were validated in R by visually checking normality of residuals, and for overdispersion and collinearity of variables.

6.3 Results

6.3.1 Does supplementary caffeine feeding affect the epidemiology of *N. bombi* in *B. terrestris* colonies?

6.3.1.i *N. bombi* infection prevalence

The covariate treatment (LMM, $F_{1,851} = 18.04$, $P < 0.001$) and the random effect colony ($P = 0.001$) both significantly reduced *N. bombi* infection prevalence in *B. terrestris* colonies. In contrast the covariates, brood cohort (LMM, $F_{1,851} = 3.36$, $P = 0.06$), number of inoculated larvae (LMM, $F_{1,851} = 0.02$, $P = 0.90$), number of days since inoculation (LMM, $F_{1,851} = 0.23$, $P = 0.63$) and the random effect forager ($P = 0.06$) all had no significant effect on *N. bombi* infection prevalence (Figure 6.1).

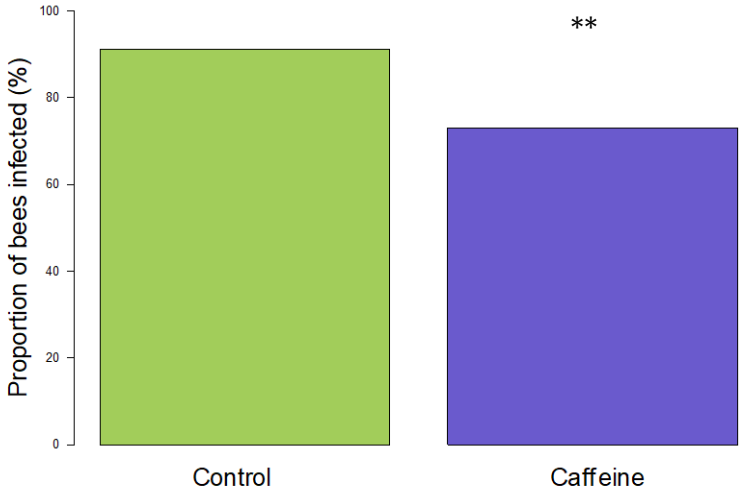


Figure 6.1 *Nosema bombi* infection prevalence in adult bumblebees (*B. terrestris*) for both control (n = 428) and caffeine (n = 272) treatments. Treatment with caffeine significantly reduced *N. bombi* infection prevalence (LMM, $F_{1,851} = 18.04$, $P < 0.001$). Significant differences have been marked with a double asterisk.

6.3.1.ii *N. bombi* infection intensity

The covariates treatment (LMM, $F_{1,851} = 34.8$, $P < 0.001$) (Figure 6.2) and cohort (LMM, $F_{1,851} = 23.6$, $P < 0.001$) (Figure 6.3) negatively affected the infection intensity of *N. bombi* in adult bumblebees. In addition there was a significant negative interaction between cohort and treatment on *N. bombi* infection intensity ($F_{1,851} = 14.2$, $P < 0.001$). In contrast the covariates, number of inoculated larvae (LMM, $F_{1,851} = 0.24$, $P = 0.6$), number of days since inoculation (LMM, $F_{1,851} = 0.001$, $P = 0.9$) and the random effects forager ($P = 0.06$) and colony ($P = 0.5$) all had no significant effect on *N. bombi* infection intensity.

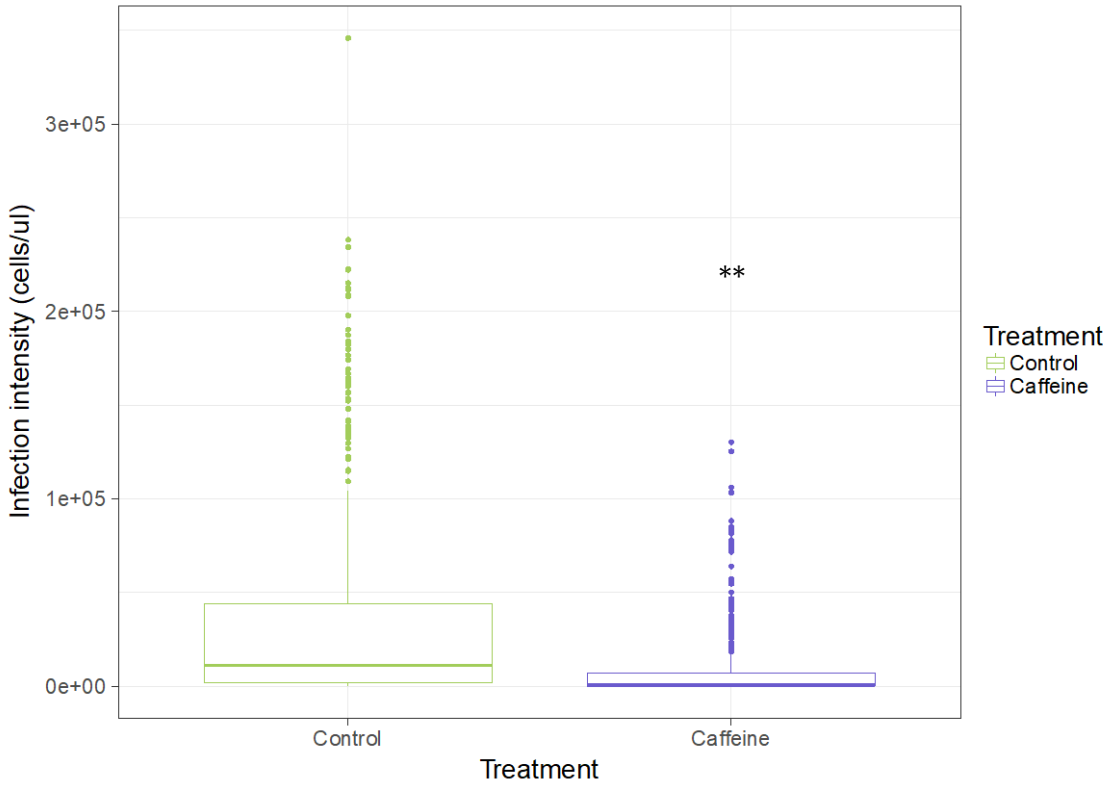


Figure 6.2 Box plot of *Nosema bombi* infection intensity in adult bumblebees (*B. terrestris*) for both control (n = 438) and caffeine (n = 272) treatments. Treatment with caffeine significantly reduced *N. bombi* infection intensity (LMM, $F_{1,851} = 34.8$, $P < 0.001$). Significant differences have been marked with a double asterisk.

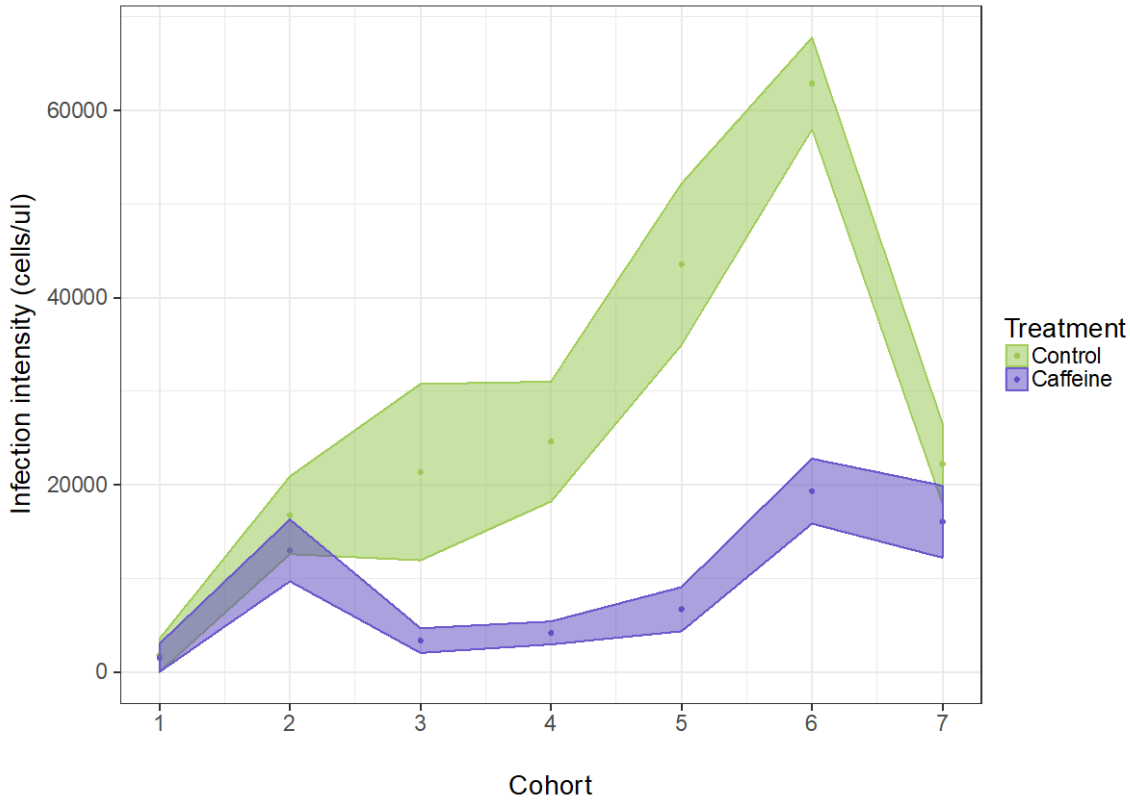


Figure 6.3 *Nosema bombi* infection intensity for specific *B. terrestris* brood cohorts. Each brood cohort is a representation of a colonies reproductive output over two weeks. For reference, younger bumblebees are in later cohorts. Cohort age (LMM, $F_{1,851} = 23.6$, $P < 0.001$) and treatment (LMM, $F_{1,851} = 34.8$, $P < 0.001$) significantly reduced *N. bombi* infection intensity in adult bumblebees at the colony terminus. In addition there was a significant negative interaction between cohort and treatment on *N. bombi* infection intensity ($F_{1,851} = 14.2$, $P < 0.001$). Shaded areas represent mean ±SEM.

6.3.2 Does supplementary caffeine feeding have an impact on bumblebee colony demographics?

The random effect colony origin significantly effected the population size attained by *B. terrestris* colonies ($P = 0.006$), with colonies varying in size from 16 to 59 adult bumblebees at colony terminus. In contrast the covariates treatment (LMM, $F_{1,18} = 2.45$, $P = 0.13$), number of brood inoculated (LMM, $F_{1,18} = 0.77$, $P = 0.3$) and number of days since inoculation (LMM, $F_{1,18} = 0.004$, $P = 0.4$) had no effect on population maxima in *B. terrestris* colonies (Figure 6.4). Similarly, the covariates treatment (LMM, $F_{1,18} = 0.96$, $P = 0.3$), population size (LMM, $F_{1,18} = 2.13$, $P = 0.17$), number of brood inoculated (LMM, $F_{1,18} = 0.04$, $P = 0.85$), days since inoculation (LMM,

$F_{1,18} = 0.032$, $P = 0.25$) and the random effect colony (P = 0.82) had no effect on the production of sexual castes (Figure 6.5).

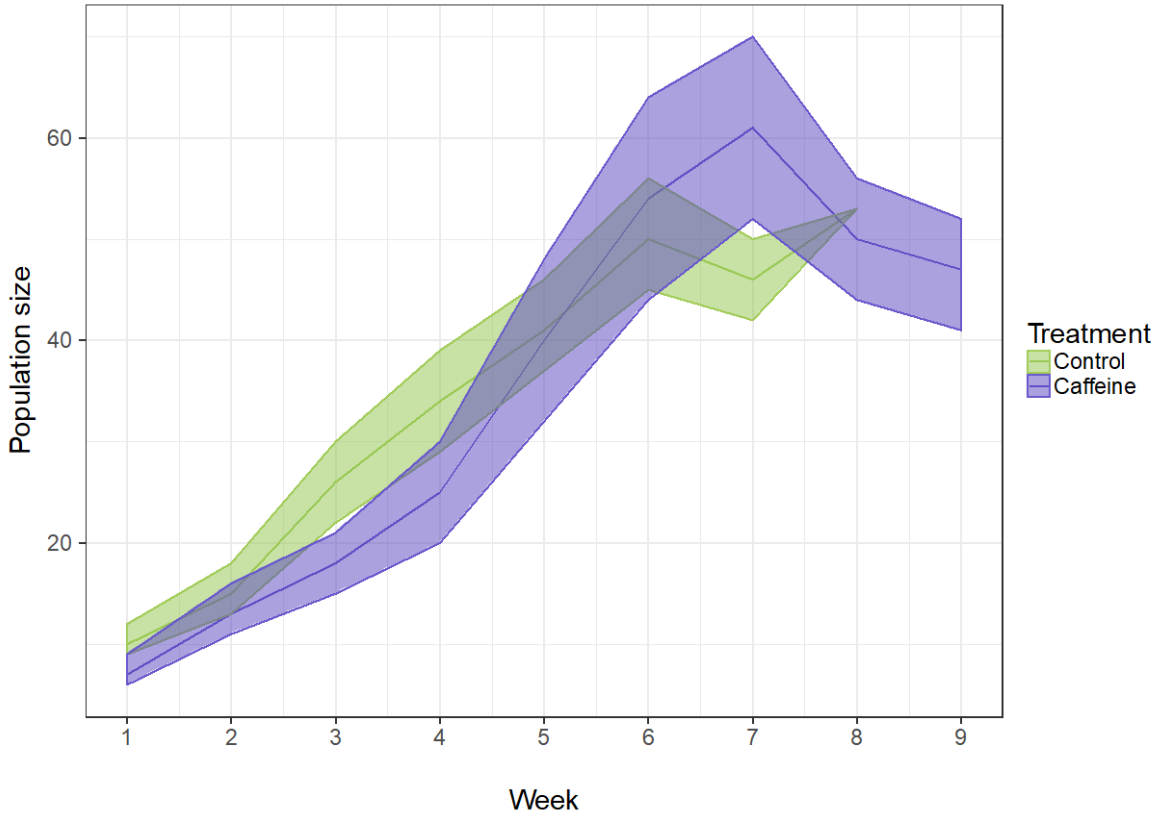


Figure 6.4 Population demographics for both control (n = 10) and caffeine (n = 9) treated *B. terrestris* colonies that were inoculated with *N. bombi*. Treatment had no significant effect on population size (LMM, $F_{1,18} = 2.45$, $P = 0.13$). Shaded areas represent mean ±SEM.

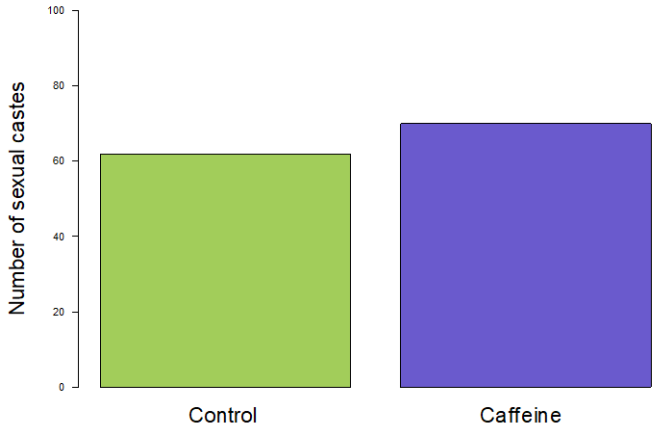


Figure 6.5 Number of sexual castes produced in control (n = 10) and caffeine (n = 9) treated *B. terrestris* colonies that were inoculated with *N. bombi*. No analysed covariates had any significant impact on production of sexual castes.

6.4 Discussion

My results show that consumption of the phytochemical caffeine, at ecologically relevant concentrations, impacted on N. bombi epidemiology by reducing the overall infection prevalence and intensity of *N. bombi* in wild caught and reared *B. terrestris* colonies. In addition these results show that chronic exposure to caffeine does not have a negative impact on bumblebee colony demographics or production of sexual castes when compared to a control group. Consequently, because caffeine is found in plants used in agri-environmental schemes, it has the potential to positively impact bumblebee health in the field.

The prevalence of EID's is increasing globally (Jones *et al*. 2008). The consequences of these epidemics on biodiversity are well documented (Daszak *et al*. 2000) and emergent fungal diseases have been linked to the population and range declines in some North American bumblebees (Cameron *et al*. 2011). Here I have shown that the methylxanthine caffeine, which was recovered from the nectar of sainfoin (*Onobrychis viciifolia*), negatively impacts the epidemiology of the microsporidian *N. bombi*, which has been implicated in bumblebee declines. Caffeine reduced the overall infection prevalence and intensity of *N. bombi in vivo*, in wild caught and reared *B. terrestris* colonies. Wild bumblebees acquire *N. bombi* presumably while foraging (van der Steen 2008), which is true of other prevalent bumblebee parasites (Durrer & Schmid-Hempel 1994, Ruiz-González *et al*. 2012). Critically *N. bombi* is infective to developing larvae and does persist though metamorphosis to the adult caste (Rutrecht & Brown 2008). Consequently a contaminated forager must return to her natal colony to vector *N. bombi* to the susceptible larval stage before infection can occur. My methodology replicated this process by artificially inoculating larvae with *N. bombi*. This ensured that each incipient colony would contain infected adult bumblebees so that *N. bombi* could propagate naturally within the colony. Interestingly microbial infection prevalence in bumblebees is dependent on both a microbes' inherent transmission potential (Otterstatter & Thomson 2007) and the parasitaemia within a given vector (Ruiz-Gonzálex & Brown 2006). Caffeine consumption reduced the overall infection prevalence of *N. bombi* with in *B. terrestris* colonies. By reducing the infection prevalence of a disease within a colony, fewer individuals are infective and subsequently fewer disease transmission events can occur (Otterstatter & Thomson

2007). In addition caffeine reduced the infection intensity in those bumblebees that were infected. Consequently, *N. bombi* may have a lower probability of successfully transmitting to a naïve host (Ruiz-González & Brown 2006). These effects on *N. bombi* prevalence and intensity, as a direct result of caffeine, impact on overall intracolonial disease epidemiology. In addition bumblebees are known interspecific vectors of disease (Meeus *et al*. 2011) and the reduction of both disease prevalence and parasitaemia in one species may also reduce the likelihood of interspecific disease transmission.

Caffeine significantly reduced *N. bombi* infection intensity across brood cohorts. This reduction suggests that chronic caffeine consumption maintains low *N. bombi* infection intensities throughout the lifespan of an adult bumblebee. In addition lower infection prevalence suggests that caffeine is impacting the ability of *N. bombi* to infect larvae. Previous work (this thesis 5.3) has shown that caffeine reduced *N. bombi* infection intensity if larvae consumed it prophylactically or therapeutically. Coupled with these results they suggest that caffeine has a negative impact on *N. bombi* replication and subsequent infection in larvae, which in turn impacts *N. bombi* epidemiology. Interestingly my results show that *N. bombi* infection intensities naturally reduce over time in control colonies. This pattern of reduced infection intensities in older, adult bumblebees has been reported in other bumblebee pathogens and is believed to be a result of a systemic immune response (Otterstatter & Thomson 2006, Rutrecht & Brown 2008). Therefore it is likely that this interaction also occurs with *N. bombi*. Nevertheless, the higher infection intensities observed in younger cohorts in control colonies were sufficient to yield higher colony infection prevalence. I suggest that this early *N. bombi* bloom in young adults, which caffeine inhibited, is critical in maintaining high intracolonial disease prevalence. High prevalence ensures that *N. bombi* can infect reproductive castes later in the colony lifecycle, which is essential in maintaining environmental prevalence within a social insect host that has an annual colony lifecycle.

Studying disease epidemiology is essential in understanding the impact of a disease on its host (Cox & Subbarao 2000). Epidemiological studies can inform robust, targeted intervention strategies that aim to mitigate the impact of disease epidemics (Ferguson *et al*. 2001, Haydon *et al*. 2006). Such interventions may make use of natural products, which have antimicrobial activity (Cowan 1999), as medicines. Interestingly, previous research has shown that secondary metabolites

found in floral rewards can impact bumblebee pathogens both *in vitro* (Palmer-Young *et al*. 2017) and *in vivo* (Manson *et al*. 2010, Richardson *et al*. 2015, Giacomini *et al*. 2018). Here caffeine was identified in the nectar of sainfoin, an angiosperm recommended in AES planting strategies to benefit pollinators (Natural England 2017). Caffeine has been reported in thirteen orders of plants (Huang *et al*. 2016) and has been shown to enhance a pollinator's memory of reward (Wright *et al*. 2013). Caffeine may therefore increase visitation rates to a flower by a foraging bee and consequently may have a strong evolutionary value for angiosperms. As described above, caffeine also reduces the prevalence and intensity of *N. bombi* infection in *B. terrestris* colonies. Consequently, plants that translocate caffeine into their floral rewards may not only gain direct fitness benefits but also indirectly benefit foraging bumblebees as they may revisit caffeine-rewarding plants (Wright *et al*. 2013), which may in turn reduce the incidence and distribution of *N. bombi* within a given environment. My results suggest that plants used in strategies that increase floral abundance and diversity, such as AES and CRP, may also indirectly benefit pollinators by impacting on disease epidemiology. The current guidelines and floral recommendations for AES have been developed with the scientific community (Pywell *et al*. 2011, Dicks *et al*. 2015) and AES have been shown to increase bumblebee species richness (Pywell *et al*. 2006, Carvell *et al*. 2007) and more recently bumblebee reproductive fitness (Carvell *et al*. 2017). By integrating epidemiological studies, such as my results, into AES, and similar global schemes their management could be further adapted to include species that have beneficial floral chemistry, which may provide indirect fitness benefits to pollinators through disease management.

Phytochemicals can have negative effects on pollinators (Cook *et al*. 2013, Tiedeken *et al*. 2016), including increased mortality in *B. impatiens* workers exposed to single phytochemicals in nectar (Richardson *et al*. 2015) and in *Apis mellifera* workers when consuming *H. annuus* pollen (Giacomini *et al*. 2018). My results show that chronic caffeine consumption did not have a negative effect on the maximum population size attained when compared to control colonies. This suggests that at the colony level caffeine is not having a detrimental effect on brood development or on queen fecundity. In addition my demographic data suggests that caffeine treated colonies had a trend for longer lifespans as they persisted longer than control colonies in the experiment. Furthermore, whilst there was a trend for a higher proportion of

sexuals to be produced in caffeine treated colonies there was no significant effect of treatment on sexual production. Consequently caffeine is not having a detrimental impact on the production of sexual castes or population density in *B. terrestris* compared to my control colonies under laboratory conditions. However, I would note that this experiment did not contain a negative control group that were not infected with N. bombi, so how caffeine impact on uninfected bumblebee demographics remains to be investigated. In addition under field conditions caffeine may manipulate pollinator fidelity (Wright *et al*. 2013) and may lead to suboptimal foraging strategies (Couvillon *et al*. 2015). Consequently plants that express caffeine in their nectar may be able to exploit the pollinator-plant mutualism, which in turn may have a negative impact on bumblebee colony fitness, not detected here, as it may lead to sub-optimal colony nutrition.

The prevalence of disease epidemics are increasing (Jones et al. 2008) and these can have severe impact on their hosts (Berger *et al*. 1998, Leopardi *et al*. 2011) including economically important pollinators (Cameron *et al*. 2011). Schemes such as AES and CRP can enhance landscapes to benefit pollinators but may also expose pollinators to a diverse suite of phytochemicals. My results suggest that these schemes and the diverse biochemistry they produce can impact on the epidemiology of important pollinator diseases. Consequently it is critical that these schemes are dynamic so that angiosperms with beneficial floral chemistry can be included as they may mitigate the impact of emerging infectious diseases in pollinators.

Chapter 7

Discussion and Conclusion

7.1 Discussion

The main aim of this thesis was to investigate the impact of phytochemicals found in the pollen and nectar of Agri-environment scheme plants on bumblebee health, more specifically to investigate whether these compounds had any impact on prevalent bumblebee parasites. The main questions addressed were:

1) Are bumblebee (*Bombus terrestris*) larvae susceptible to *C. bombi* infection and can the developing brood act as a disease transmission hub? (*Chapter 2*)

2) What phytochemicals are found in the pollen and nectar of plants included in UK based Agri-environment schemes (AES), and do these have an impact on the *in vitro* growth of *Crithidia bombi*? (*Chapter 3*)

3) Do AES phytochemicals impact on the infection prevalence and intensity of the microsporidian *Nosema bombi in vivo*? (*Chapters 4 & 5*)

4) Do AES phytochemicals impact on the intracolonial epidemiology of the microsporidian *Nosema bombi*? (*Chapter 6*)

The focal host species used in my experiments was the buff-tailed bumblebee (*Bombus terrestris*). This generalist bumblebee species has a relatively large geographic range, and a high population density within the UK. Bumblebees, such as *B. terrestris*, are exposed to a range of microbial parasites (Schmid-Hempel 1998), which may have negative impacts on the developing colony (e.g. Brown *et al*. 2003, Otti & Schmid-Hempel 2007). Consequently, it was critical that my experiments investigate not only the interaction between phytochemicals and bumblebee parasites at different host developmental stages but also attempt to elucidate the impact of phytochemicals on parasites in individual bees and on the colony as a whole.

7.2 Key findings

7.2.1 Transmission dynamics of Crithidia bombi (*Folly et al. 2017 Journal of Invertebr Pathol*)

Crithidia bombi (trypanosomatidae) (Gorbunov 1987) has a relatively high environmental prevalence and can reduce bumblebee colony fitness by up to 40% (Brown *et al*. 2003). Prior to this thesis it was unclear whether *C. bomb*i could infect *B. terrestris* larvae, and if infection was successful, whether there were any negative fitness consequences for the developing brood. My novel results, gained from artificial inoculation of developing *B. terrestris* brood, show that larvae do not become infected with *C. bombi* seven days after inoculation. It is likely that bumblebee larval guts have a high osmotic potential due to the sugar-heavy feeding regime they receive from brood caring workers (Pereboom 2000), which may in turn be unsuitable for *C. bombi* development. This was an important finding for the design of subsequent chapters that focused on the impact of phytochemicals on bumblebee parasites. The reason for this is that the primary protein source for developing bumblebee larvae is pollen (Sladen 1912, Wilson 1972, Alford 1978). Whilst both pollen and nectar contain phytochemicals (Adler 2000, Stevenson *et al*. 2017), typically pollen contains a higher concentration of phytochemicals when compared to nectar (Adler 2000). Consequently, during trophallaxis larvae may be exposed to higher concentrations of phytochemicals when compared to workers, who consume predominantly nectar, and this may be important for understanding interactions with parasites. However, as I showed that larvae are not susceptible to *C. bombi* infection, experiments that are designed to test the impact of pollen phytochemicals on infected bumblebee brood need to use a parasite that is known to infect *B. terrestris* brood, such as the microsporidian *Nosema bombi*. Thus, these results determined the host-parasite system for most of the rest of the thesis.

Microbial parasites are often dependent on contact networks to facilitate transmission between hosts (Otterstatter & Thomson 2007). The trypanosome *C. bombi* is presumably transmitted between adult intercolonial conspecifics at shared resources, such as flowers (Durrer & Schmid-Hempel 1994, Ruiz-González *et al*. 2012, Adler *et al*. 2018), before being vectored to a naïve colony. Once the parasite is established within a colony, intracolonial transmission is dependent on contact

networks (Otterstatter & Thomson 2007). Whilst my results show that *B. terrestris* larvae cannot become infected with *C. bombi*, prior to my work whether larvae could act as disease transmission hubs had not been investigated. There are multiple feeding interactions between workers and the developing brood that take place within a bumblebee colony (Katayama 1973) and these may facilitate parasite transmission. My results, from chapter two, show that these feeding interactions are sufficient to vector *C. bombi* from a naïve worker who has consumed a contaminated food source, via trophallaxis with a developing larvae, to another naïve worker, resulting in the infection of the second worker. Identifying this dynamic means that larvae can now be integrated into bumblebee disease networks where contact between individuals is an important predictor of infection risk. Consequently, this could have important implications for understanding disease transmission in social insect colonies more generally.

The results from chapter two indicate that all members of a social insect colony, regardless of developmental stage, may have an impact on microbial disease transmission. Further research integrating the spatial fidelity of bumblebee workers (Crall *et al*. 2018), more specifically brood caring workers and foragers, would be useful in understanding how division of labour relates to disease transmission. Given that other social insects have structured contact networks, which may protect vulnerable members of a colony (Naug & Camazine 2002, Naug 2008), it is likely that spatial fidelity in bumblebees may be an important indicator of intracolonial disease risk.

7.2.2 Floral chemistry of Agri-environment schemes and its effect on *Crithidia bombi in vitro*

Agri-environment scheme (AES) planting strategies have been designed to provide a mix of angiosperms that are thought to benefit pollinators (Dicks *et al*. 2015). In addition, florally enriched landscapes have been shown to increase bumblebee fitness (Carvell *et al*. 2017). Indirectly these schemes also increase the diversity of phytochemicals that pollinators are exposed to. My results from chapter three identified 62 phytochemicals, using LC-MS, in the pollen and nectar of AES plants

that bumblebees are known to forage on. Given that foraging bumblebees undertake numerous foraging trips it is likely that a developing colony is continuously exposed to a diverse suite of phytochemicals. Consequently, determining the chemical profile of forage plants is key to understanding how landscapes and schemes such as AES may impact on pollinator health.

I then investigated the impact of four AES phytochemicals on *C. bombi* growth, *in vitro*. The plant hormone abscisic acid and the isoflavone biochanin A both had no significant effect on *C. bombi* growth when compared to a control group. However, the alkaloid caffeine and the polyamide tricoumaroyl spermidine both had a significant effect on *C. bombi* growth. Tricoumaroyl spermidine, which was recovered from the pollen of birds-foot trefoil (*Lotus corniculatus*), only reduced *C. bombi* growth in one of the two parasite strains at five times its ecologically relevant concentration. Whilst tricoumaroyl spermidine has reported biological activity (Walters *et al*. 2001), it is likely that *C. bombi* has evolved to negate the impact of this common pollen constituent (Palmer-Young *et al*. 2017). In contrast, caffeine at its ecologically relevant concentration increased the *in vitro* growth of *C. bombi*. However, at higher concentrations, which are still ecologically relevant, for example in citrus plants (Kretschmar & Baumann 1999), caffeine inhibited *C. bombi* growth. This is an important and novel result as it clearly highlights that a single phytochemical may have different impacts on pollinator health depending on what concentration is encountered during foraging. I would note that these results are from an *in vitro* investigation, and how phytochemicals impact on disesaes such as *C. bombi* may be different in an *in vivo* experiment and these differences may arise as a consequence of host immune response.

Previous research into the impact of phytochemicals on *C. bombi* growth *in vitro* have used optical density as a proxy for parasite growth (Palmer-Young *et al*. 2016, Palmer-Young *et al*. 2017). My results are the first, to my knowledge, that combine optical density data with parasite count data to provide a holistic overview of *in vitro C. bombi* growth. Using these separate measurements I was able to elucidate the impact of phytochemicals on different *C. bombi* strains that were not identified using optical density alone. In addition, parasite count data may be a more important measurement when assessing the impact of phytochemicals on bumblebee health, as it is a critical factor that determines infection success (Ruiz-González & Brown 2006).

In the wild bumblebees typically have a broad diet (Sladen 1912). Consequently even in AES enhanced land they may be exposed to bioactive phytochemicals, which may have important implications for bumblebee health, that were not investigated in this chapter. Bumblebees typically feed directly via trophallaxis, and this interaction may contain pollen and nectar from several species of flower. Given that phytochemicals may interact (Palmer-Young *et al*. 2017) and that pollen from a single species of flower may have multiple benefits for pollinator health (Giacomini *et al*. 2018) I believe it is critical to investigate the chemical profile of pollen loads brought to the colony by returning foragers. This would provide a more realistic profile of the phytochemistry that bumblebees are exposed to under natural field settings. Equally as important, comparative studies, which investigate pollen loads in florally enriched, compared with depauperate landscapes would be useful in understanding into how schemes that increase floral abundance and diversity, such as AES, affect the chemical landscape.

7.2.3 The effect of biochanin A on *Nosema bombi in vivo*

Following my literature review (Chapter 1) I was determined to design a range of experiments that investigated the impact of phytochemicals on *Nosema bombi*. This microsporidian parasite has lower environmental prevalence (Shykoff & Schmid-Hempel 1991, Jones & Brown 2014) but arguably higher virulence than *C. bombi* (Otti & Schmid-Hempel 2007, Rutrecht & Brown 2009, Cameron *et al*. 2011). To my knowledge, prior to this thesis, there was no research on the impact of phytochemicals on *N. bombi*. This was primarily due to a lack of robust methodology for individual bumblebee inoculations. The most advanced methods were described in Rutrecht & Brown (2007) where *B. lucorum* colonies were inoculated with *N. bombi* by contaminating pollen pellets. However, to elucidate the impact of phytochemicals on *N. bombi* in individual bees meant designing a protocol that could successfully inoculate individual developing larvae with a predetermined concentration of *N. bombi*. Following eclosure adult bees could then be screened via microscopic examination of faeces to investigate the impact of phytochemicals. My novel methodology, in chapter four, enabled the quantifiable inoculation of individual

larvae using a pipette. As *N. bombi* can infect larvae and the infection persists through eclosure this enabled the design of novel bioassays that could investigate the impact of phytochemicals on infection in different bumblebee developmental stages. Firstly, inoculating larvae with *N. bombi*, pre or post-phytochemical exposure, investigated the potential prophylactic or therapeutic effect of a phytochemical. Secondly, exposing an infected adult worker to a phytochemical for a period of seven days allowed the detection of any therapeutic impact on infection intensity. As these methods and the concept of phytochemicals impacting *N. bombi* infection in bumblebees were novel, I opted to conduct a proof of principle investigation.

Biochanin A was identified in the nectar of red clover (*Trifolium pratense*) at a concentration of 0.1ppm (Chapter 3). This isoflavone has reported biological activity against fungal microbes as it can compete for cell wall receptor sites (Weidenbörner *et al*. 1990). To increase the likelihood that an effect would be detected in my bioassays I tested the impact of biochanin A at 20ppm on *N. bombi* infection in larvae and adults. It is important to note that a concentration of 20ppm is still within the realistic concentration range of *T. pratense* floral reproductive tissue (Saviranta *et al*. 2008). My results show that *N. bombi* infection intensity was significantly reduced in adult bumblebees that were prophylactically fed biochanin A as larvae. In addition, my results also show that biochanin A had a significant therapeutic effect in infected adult bumblebees. Both of these results clearly demonstrate that my novel inoculation protocol was successful and that phytochemicals may have a significant impact on *N. bombi* infection in different bumblebee developmental stages.

As described above, the microsporidian *N. bombi* has arguably higher virulence when compared to other prevalent bumblebee parasites. The development of a quantifiable *N. bombi* inoculation protocol opens up a number of imperative host-parasite questions, which may now be addressed. Firstly, what is the threshold level at which infection occurs, and is this species or caste specific? This question may have important implications for our understanding of intercolonial transmission between congeners, and ultimately *N. bombi* environmental prevalence. Secondly, findings on the virulence of *N. bombi* have been inconsistent (Fisher & Pomeroy 1989, Whittington & Winston 2003, Otti & Schmid-Hempel 2007, Rutrecht & Brown 2009, Cameron *et al*. 2011). Using ecologically relevant, quantifiable doses and tracking infection would provide a greater understanding on the impact of field realistic *N. bombi* virulence in bumblebees.

The results from chapter three demonstrate that the floral chemistry of plants used in AES may improve wild bumblebee health. Consequently, by maintaining beneficial wildflower strips, landowners may indirectly improve angiosperm based crop yields by supporting the health of key insect pollinators.

7.2.4 The effect of caffeine and tricoumaroyl spermidine on *Nosema bombi in vivo*

Following on from the success of chapter four, the next logical step was to investigate the impact of AES phytochemicals at their ecologically relevant concentration on *N. bombi in vivo*. As both caffeine and tricoumaroyl spermidine have known biological activity (Raj & Dhalla 1965 and Walters *et al*. 2001, respectively), including against bumblebee parasites (Chapter three), they were selected to be trialed in bioassays using developing *B. terrestris* larvae. My results show that tricoumaroyl spermidine had no significant prophylactic or therapeutic effect on *N. bombi* infection prevalence or intensity. However, the infection intensity of *N. bombi* was significantly reduced in adult bumblebees that were fed caffeine either prophylactically or therapeutically as larvae. These results provide the first data on the positive impact of phytochemicals, at ecologically relevant concentrations, on *N. bombi* infection in *B. terrestris*.

AES planting strategies have been developed with the scientific community in an attempt to ensure they are beneficial for pollinators (Dicks *et al*. 2015). Research has shown these schemes may increase bumblebee abundance (Pywell *et al*. 2006, Carvell *et al*. 2007) and more recently fitness (Carvell *et al*. 2017). My results highlight for the first time that the floral chemistry of plants included in AES planting strategies may also indirectly impact on pollinator health by mitigating the impact of an important bumblebee pathogen. In addition, my results suggest that dynamic schemes such as AES and its American counterpart the Conservation Reserve program (CRP) could be tailored to include plants with beneficial floral chemistry to provide indirect health benefits to pollinators.

7.2.5 Effect of caffeine on the epidemiology of *Nosema bombi*

The previous three chapters focused on the effect of phytochemicals on *in vitro* trypanosome growth or on microsporidian infection intensity in individual bumblebees. However, for my final chapter it was imperative to investigate the impact of phytochemicals on overall colony health. Bumblebees form small relatively simple, annual colonies (Wilson 1971) and diseases can reduce overall colony fitness (e.g. Brown *et al*. 2003). Epidemiological studies are critical in understanding diseases (Cox & Subbarao 2000, Keeling *et al*. 2001), and may facilitate the design of disease intervention strategies (Haydon *et al*. 2006). My novel results using wild caught and reared *B. terrestris* colonies show that continuous caffeine consumption significantly reduced the overall colony infection prevalence of *N. bombi*. In addition, those bumblebees that were infected had significantly lower *N. bombi* infection intensities across temporally different brood cohorts when compared to control groups. These results clearly show that the consumption of phytochemicals at ecologically relevant concentrations has the potential to positively impact bumblebee health and may also impact on pollinator disease epidemiology. This in turn may also impact on crop yields in AES enhanced landscapes as diseased bumblebees make inefficient foragers and produce smaller colony sizes. By mitigating the impact of diseases through schemes such as AES landowners may support biodiversity whilst indirectly improving crop yields.

Phytochemicals with biological activity have been shown to have negative effects on pollinators (Cook *et al*. 2013, Tiedeken *et al*. 2016). However, my results show that chronic exposure to caffeine had no significant negative impact on either total colony population or on production of sexual castes. I would note however that during the lifecycle of a colony, bumblebees are continuously exposed to a diverse suite of phytochemicals, which may have differing impacts on colony fitness (Arnold *et al*. 2014, Palmer-Young *et al*. 2016). Consequently, understanding how these compounds may interact is crucial in determining how the chemical landscape truly impacts on pollinator health.

The results from chapter five and six provide the first evidence that naturally encountered phytochemicals may impact bumblebee health and disease epidemiology. I would note however that these results were obtained under laboratory conditions and

how they relate to the field realistic foraging strategies of bumblebees when faced with a parasite challenge remains unclear. Given that species-specific pollen isolates can negatively impact on disease in bumblebees (Giacomini *et al*. 2018) and that nutrition is critical in bumblebee immunocompetence (Brown *et al*. 2000, Brunner *et al*. 2014) it is likely that an interaction between resource quality and floral phytochemistry may be a critical component of field realistic disease epidemiology in bumblebees. Consequently, field-based bumblebee epidemiological studies should be a priority for researchers.

7.3 Other directions for future research

Within this thesis I have investigated several key interactions between phytochemicals, found in the pollen and nectar of AES plants, and bumblebee parasites. Below I briefly discuss several other related areas of research that I believe to be important, given my results, and for which relatively little is currently known.

7.3.1 Floral chemistry of plants not included in Agri-environment schemes, including economically important crops

I have comprehensively screened, using LC-MS, the pollen and nectar from all plants included in AES that bumblebees are known to forage on. Given that the phytochemistry of AES plants has the potential to positively impact bumblebee health (Chapters 3, 4, 5 & 6) it seems highly probable that species not currently included in AES planting strategies, may have beneficial floral chemistry that may indirectly improve bumblebee health. Investigating the chemistry of such plants may provide policy makers with additional information when designing beneficial planting strategies so that they effectively support pollinator health.

In addition to the recommendation above, investigating the phytochemistry of economically important flowering crop species, which typically have a short anthesis

period, may mean that field margins could be individually tailored to provide positive health benefits to bumblebees throughout the lifecycle of a colony. For example, in relation to my findings, areas of high *N. bombi* prevalence could contain a greater number of caffeine producing plants. However plants that produce caffeine may manipulate pollinators into sub-optimal foraging decisions (Wright *et al*. 2013, Couvillion *et al*. 2014). Consequently, supplementary flowers that do not contain caffeine, and which have a different flowering period to caffeine producing species, could be included to ensure that foraging bumblebees and ultimately developing colonies receive adequate nutrition, whilst maintaining the potential health benefits of caffeine. Understanding and effectively designing the composition of species included in planting strategies to benefit pollinators should be a priority for researchers, conservationists and government policy makers alike.

7.3.2 Self medicative behaviour

There is increasing evidence across the animal kingdom for self-medicative behaviour using plant secondary metabolites (Huffman 2001, de Roode *et al*. 2013, Abbott 2014). Examples of self-medicative behaviour from across the phylum Arthropoda are similar because the compounds that are collected when faced with a parasite challenge are considered part of a normal diet instead of novel food sources (Povey *et al*. 2009, Abbott 2014). Identifying if bumblebees can adapt and alter their forage preferences when faced with a parasite challenge may provide stimulating evidence to the concept of social immunity. Primarily I recommend that this concept be investigated under laboratory conditions, where infected bumblebees would be provided access to control or experimental nectar. During the experimental timeframe, I recommend a record of each bumblebee's daily nectar consumption and infection intensity be taken. If it was shown that infected bumblebees had a preference for the experimental nectar it may suggest that they are self-medicating at an individual level. This methodology would then need to be developed to ascertain whether individual foragers are making foraging decisions that may benefit the colony. More specifically, again under laboratory conditions, I recommend providing infected and non-infected colonies with a control and experimental nectar supply, as this would investigate colony level foraging preferences. In conjunction with this, I recommend integrating my methodology from chapter six of this thesis, which may

elucidate an impact on intracolonial disease epidemiology as a direct result of foraging preferences.

7.4 Conclusion

Wildlife disease epidemics are increasing globally and these can have negative fitness consequences for their hosts (Berger *et al*. 1998, Jones et al. 2008, Leopardi *et al*. 2011). Bumblebees provide essential pollination services (Breeze *et al*. 2011, Kleijn *et al*. 2015) and there is evidence to suggest that these charismatic insects are undergoing both range and population declines (Williams 1982, Fitzpatrick *et al*. 2007). These declines are due, in part, to an increase in disease prevalence and a reduction in wildflower meadows. Schemes that aim to support biodiversity by increasing floral abundance and diversity may also provide pollinators with indirect fitness benefits through the mitigation of diseases. My research has identified that current Agri-environment scheme (AES) planting strategies may positively impact bumblebee health. In addition, my research has highlighted that identifying and integrating plants, with good nutritional resources and beneficial phytochemistry, into strategies that increase floral abundance and diversity to benefit pollinators, should be a priority for policy makers, as these may improve pollinator health and consequently improve crop yields.

Bumblebees are an intriguing and captivating component of the natural world. They, as many species, are under threat from emerging disease epidemics. Understanding how such diseases can be mitigated or managed provides a route by which we as humans may attempt to preserve and support our planet's wonderful and unique biodiversity.

8. References

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9. Appendices

Appendix 1.1 Methodology designed by Iain W. Farrel for the synthesis of Tricoumaroyl spermidine (*used in chapter three of this thesis*)

Tricoumaroyl spermidine synthesis protocol

H&S: all stages must be carried out in a well-ventilated fume cupboard dedicated to this protocol for the duration of the synthesis.

Personnel to wear gloves, eye protection at all times.

In the event of spillages, use copious amounts of cold tap water to dilute the spillage.

In the event of inhalation of fumes, leave the laboratory and seek medical attention.

Stage 1 acetylation of (E-)-4- hydroxypropenoic acid

Apparatus: Measuring cylinder (50 mL) 100 mL conical flask, magnetic flea, hotplate stirrer, weighing bottles, spatula, balance (0.1g),

500 mL beaker, small Büchner funnel, vacuum pump, desiccator (dried).

Time: three days from start to dried product.

1. Place pyridine (40mL) in a 100 mL conical flask + stopper with magnetic flea on a hot plate stirrer.
2. Add coumaric acid (4.0g, 24.4 mmol) and wait until all has dissolved.
3. Add slowly, no more than 5 mL at a time, acetic anhydride (40 mL, 363 mmol, large excess).
4. Leave stirring overnight (approx. 16h).
5. Slowly pour the reaction mixture into a 500 mL beaker containing ice-cold distilled water (400 mL) and stir to encourage precipitation of the acetylcoumaric acid.
6. Collect the product by filtration using a small Büchner funnel and vacuum filtration.
7. Wash with ice-cold distilled water until the filtrate no longer smells of acetic acid or pyridine.
8. **Dry the product thoroughly** in a Rotovap and then overnight in an oven at 100°C and then store in a desiccator.
9. Weigh and calculate yield (lit. 81%, approx. 4 g).
10. Check a sample by LCMS against coumaric acid looking for (+) m/z = 207

[Lit.: *To a solution of coumaric acid (2g, 12.2 mmol) in pyridine (20 mL) was added acetic anhydride (20 mL, 181 mmol) and stirred for 16 h. The product was precipitated by pouring onto water (200 mL) and collecting by filtration a white powder (2.02 g, 9.9 mmol, 81%).]*

Chemical	**MF**	**MW**	**mass/g**	**mmol**	
p-coumaric acid	C9H8O3	164	4.0	24.4	yield based on this
acetic anhydride	C4H6O3	102	37.04	363	large excess
pyridine	C5H5N	79	39.28	497	vast excess
acetylcoumaric acid	C11H10O4	206	4	19.4	80%

Stage 2 chlorination of acetylcoumaric acid to (E)-4-O-acetylcoumaroyl chloride.

Assuming a yield of 4g from stage 1.
Apparatus: measuring cylinder (25 mL), round bottomed flask (weighed, 100 mL), magnetic flea, hot plate stirrer, spatula, desiccator (dried)
Time: 1 day plus overnight drying.

1. Place oxalyl chloride (25gL, 197 mmol, b.p. 61^{o}C) in a dried, weighed round bottomed flask (100 mL) with a magnetic flea on a stirrer.
2. **Slowly** add E-4-O-acetylcoumaroyl chloride from stage 1 (4.0 g, 19 mmol) using a spatula.
3. Stir for 3h
4. Evaporate to dryness on a Rotovap and leave in a desiccator overnight.
5. Weigh to determine approximate yield (Lit. 100%, 4.0g)

[Lit.: (E)-4-O-acetylcoumaroyl chloride. A solution of (*E*)-4-O-acetylcoumaric acid (500 mg, 2.4 mmol) in oxalyl chloride (4.3 mL, 50 mmol) was stirred for 3 h. The resulting mixture was evaporated to dryness to yield the product as a white powder (545 mg, 2.4 mmol, 100%)}

Chemical	**MF**	**MW**	**mass/g**	**mmol**	
acetylcoumaric acid	C11H10O4	206	4.27	19.4	80%
oxalyl chloride	C2O2Cl2	127	13.33333	105	5xexcess
acetylcoumaroyl chloride	C11H9O3Cl	224.5	4.36	19.4	100%

LCMS chromatogram

(+) m/z = 207 = unchanged acetylcoumaric acid

(+) m/z = 225 and 227 (3:1) = product, acetylcoumaroyl chloride

Stage 3: acylation of spermidine by product of stage 2.

Assuming a yield of approx. 4.0 g from stage 2.

Proceed at approx. x 8 scale of Lit. protocol.

Dry the THF (anhydrous sodium sulphate). **Distil** if necessary to obtain pure, dry THF.

Prepare brine in advance (overnight).

Prepare 5% and 10% methanol in dichloromethane for flash chromatography.

Apparatus: measuring cylinder (25 mL), conical flask x 2 (50 ml), magnetic fleax2 and hot plate stirrer, separating funnel (250 mL), measuring cylinder (50 mL), conical flask (250 mL) round bottomed flask (250 mL for ethyl acetate fractions), round bottomed flask (500 mL) for ethyl acetate evaporation,

Vacuum flash chromatography kit.

Time: two to three days

1. Add a portion of THF (25 mL) to the product obtained from stage 2 (4.40g, 19.6 mmol, *E*-4-O-acetylcoumaroyl chloride) and stir until dissolved.
2. Place THF (25 mL) in a dried conical flask (50 mL) with a magnetic flea on a hot plate stirrer.
3. Add spermidine (800 mg, 875 uL, 5.6 mmol) to the THF.
4. Pipette trimethylamine (3.0 mL, 21.8 mmol, an excess of all likely HCl) into this mixture.
5. To the solution in step 4 add, dropwise using a teat pipette, the solution prepared at the end of step 1. A white precipitate forms and the reaction mixture turns yellow then orange.
6. Leave stirring overnight. Check progress by LCMS: 10% dilution, spun at 13,000 rpm for 10 min.
   ++
7. In a separating funnel (glass or Teflon taps) place brine (100 mL),
8. Add the reaction mixture from step 7.
9. Add ethyl acetate (100mL) and extract.
10. Repeat extraction of the brine with two more portions (100 mL) of ethyl acetate.
11. Add methanol to dissolve any suspended solids (approx. 50 mL).
12. Dry combined extracts over anhydrous magnesium sulphate (30 min).

13. Filter using a Buchner fuller into a weighed round bottomed flask (500 mL).
14. Evaporate to dryness on a Rotovap. Product is a yellow solid.
    ++
15. Redissolve product in a minimum volume of methanol (2 ml?)
16. Prepare 5% methanol in dichloromethane and 10% methanol in dichloromethane. (1 L each).
17. Run flash chromatography under reduced pressure in a fume cupboard (old kit), collect two fractions of 40 mL each and run samples on LCMS to check purity. Looking for m/z =710.
18. Evaporate fractions with this MW to dryness on the Rotovap (**chlorinated waste**).
19. Weigh to determine yield. (Lit: 73% = 3 g)

[Lit. N^1,N^5,N^{10}-Tri((E)-4-O-acetylcoumaroyl) spermidine. To a solution of (*E*)-4-O-acetylcoumaroyl chloride (279 mg, 1.24 mmol) in THF (25 mL) was added dropwise a solution of spermidine (50 mg, 0.35 mmol) and triethylamine (250 μL) in THF (25 mL). The mixture was stirred for 16 h before brine (35 mL) was added. The organics were extracted into ethyl acetate (3 × 50 mL) dried over $MgSO_4$ and the solvent removed *in vacuo*. The residue was purified using flash chromatography (5-10% methanol in DCM) to yield the product as a white powder (180 mg, 0.25 mmol, 73%)]

Chemical	**MF**	**MW**	**mass/g**	**mmol**	
spermidine	C7H19N3	145	0.8	5.61	yield based on this
acetylcoumaroyl chloride	C11H9O3Cl	224.5	4.4	19.6	3.5x excess over spermidine
triethylamine	C6H15N	101	2.2	21.6	Excess over HCl
trisacetylcoumaroylspermidine	C40H43N3O9	709	2.98	4.2	expected

LCMS chromatogram expected peaks

(+) m/z	**ID**	**Notes**	**RT/min**
146	spermidine	Unreacted starting material	Not seen
225 and 227 (3:1)	acetylcoumaroyl chloride	Unreacted, excess reagent	13.73
334	monoacetylcoumaroylspermidine	Incomplete reaction product	5.05

522	diacetylcoumaroylspermidine	Incomplete reaction product	12.8
710	trisacetylcoumaroylspermidine	Final reaction product	18.0

compound	**Peak area (MA)**	**Relative peak area**
monoacetylcoumaroylspermidine	353430	1%
diacetylcoumaroylspermidine	17686349	3%
trisacetylcoumaroylspermidine	488596606	96%

Stage 4 hydrolysis of acetylated triscoumaroyl spermidine

Assume 1.40 g yield from stage 3.
Apparatus: conical flask (250 mL), conical flask (100 mL), measuring cylinder (25 mL), magnetic flea, hot plate stirrer,
Time: 1 -2 days including drying.

1. In a conical flask (100 mL) prepare a 0.1 M solution of KOH (0.27 g, 100 mmol) in methanol (50 mL). Leave to cool.
2. Into a conical flask (250 mL) place methanol (25 mL) and THF (25 mL).
3. Add all the product from stage 3 (est. 1.4 g, 2.0 mmol).
4. Stir for 3 h.
5. Add HCl (5 ml conc in 25 mL water) to neutralise the reaction mixture (check with pH paper).
6. Collect filtrate in a small Böchner funnel and wash with ice-cold distilled water until the filtrate is neutral to litmus.
7. Combine the filtrate and extract this with diethyl ether (3x 50 mL) in a separating funnel.
8. Dry combined extracts over anhydrous sodium sulphate (30 min).
9. Filter or preferably decant into a round bottomed flask (250 mL) in two batches.
10. Transfer to a weighed specimen bottle using methanol.
11. Dry off (nitrogen line) & reweigh to determine the yield. (Lit 99% expect 1.1g)

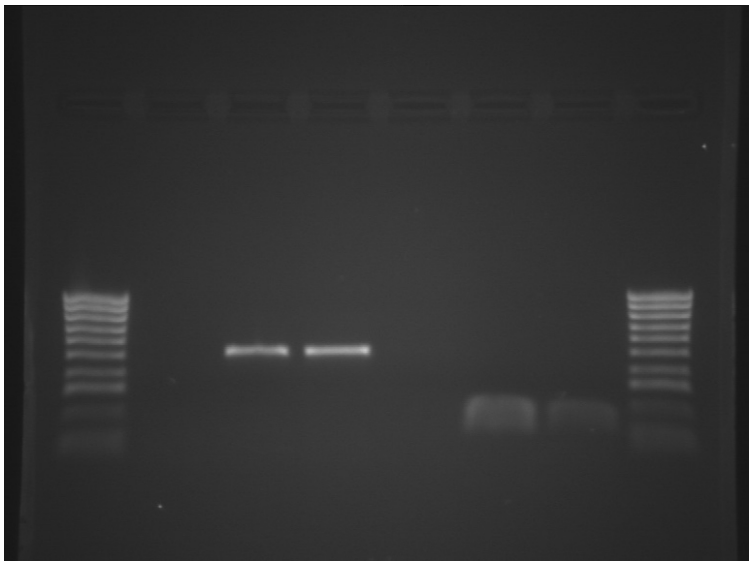
[Lit. N^1,N^5,N^{10}-Tri-p-coumaroyl spermidine. To a solution of N^1,N^5,N^{10}-Tri((E)-4-O-acetylcoumaroyl) spermidine (180 mg, 0.25 mmol) in Methanol-THF (1:1, 50 mL) was added a solution of 0.1 N KOH in methanol (50 mL). The resulting mixture was stirred for 3 h after

which it was neutralised by the addition of a 0.1N HCl solution. The white precipitate was removed by filtration and the filtrate was extracted with diethyl ether (3 × 50 mL). The organics were combined, dried over $MgSO_4$ and the solvent removed *in vacuo,* yielding the product as off white crystals (147 mg, 25 mmol, 99%).]

Chemical	**MF**	**MW**	**mass/g**	**mmol**	
trisacetylcoumaroylspermidine	C40H43N3O9	709	1.418	2.0	expected
KOH	KOH	54	0.27	5	2.5xexcess
HCl	HCl	36.5	0.183	5.0	35% w/v = 10M
triscoumaroylspermidine	C34H37N3O6	583	1.17	2	expected

Appendix 1.2 PCR amplicons of *N. bombi*, amplified using n.b.a primer pair (Erler *et al*. 2011) (expected amplicon length 511 bp), resolved in 1.5% agarose gel and stained with ethidium bromide. (L= 100bp marker, A= my inoculum, B = *N. bombi* positive control).

L A B



Appendix 1.3 *Bombus terrestris* colony data for epidemiology investigation (*chapter five*).

Colony #	Date of queen catch	Date of first clutch	# Larvae inoculated	Treatment
17	15.02.18	03.05.18	14	Caffeine
9	22.02.18	30.04.18	14	Caffeine
29	21.02.18	24.05.18	13	Caffeine
6	14.03.18	22.04.18	14	Control
20	13.03.18	08.05.18	11	Control
25	14.03.18	18.05.18	14	Caffeine
2	07.03.18	09.04.18	14	Control
18	14.03.18	03.05.18	14	Control
19	14.03.18	18.05.18	12	Caffeine
27	13.03.18	04.05.18	14	Control
30	22.02.18	18.05.18	14	Control
16	07.03.18	24.05.18	12	Control
28	22.02.18	04.05.18	13	Control
5	14.03.18	24.05.18	14	Caffeine
14	14.03.18	15.04.18	13	Control
22	13.03.18	30.04.18	14	Control
31	13.03.18	27.05.18	13	Caffeine
7	16.03.18	22.04.18	14	Caffeine
32	08.03.18	29.05.18	14	Caffeine

10. Final acknowledgements

'So long and thanks for all the fish' (Adams 1984)